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14. ABSTRACT
Purpose: The goal of the project is to identify the molecular mechanisms responsible for therapeutic failure in prostate cancer patients receiving hormonal therapy Scope: The scope of the project is to use prostate cancer cell lines in in-vitro cell culture systems to study the complex signal transduction pathways that may be responsible for the neuroendocrine differentiation of prostate cancer cells, particularly the relationship of PTP1B to IL-8 signaling through its receptors CXCR1 and CXCR2, to IGF-1 receptor signaling through PI3 kinase/AKT/mTOR pathway and to androgen receptor signaling. Major findings: We have established LNCaP cell lines stably expressing CXCR1 and CXCR2. In such cell lines, PTP1B is overexpressed when compared with parental cells. There is also significant increase in the expression of NSE, a marker of neuroendocrine differentiation, in such stable cell lines. We studied the expression of IL-8 in LNCaP cells stably overexpressing PTP1B and a mutant PTP1B and found increased levels of IL-8 in such cell lines in comparison to parental LNCaP cells. Expression of IL-8 receptors CXCR1 and CXCR2 are also increased in these stable cell lines. A related but unexpected significant finding is that the neuroendocrine cells may be the stem cells of prostate cancer.

15. SUBJECT TERMS
Prostate cancer, hormonal therapy, neuroendocrine cells, PTP1B, IL-8, CXCR1, CXCR2, IGF-1 receptor, androgen receptor, cancer stem cells

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Introduction:

The ultimate goal of our research is to study the molecular mechanisms responsible for therapeutic failure in prostate cancer (PC). PC, when localized, can be treated with surgery or radiation. Once the tumor is advanced or metastatic, hormonal therapy, consisting of androgen ablation and/or anti-androgen, is the treatment of choice. Unfortunately, although all patients respond to this treatment initially, the tumor invariably recurs and enters the androgen-independent (hormone-refractory) stage, for which there is no effective therapy. We hypothesize that the reason for the eventual failure of the therapy is because PCs contain neuroendocrine (NE) cells in addition to the more abundant secretory type cancer cells. Unlike the secretory type cancer cells that express androgen receptor (AR) and depend on androgen for proliferation, NE cells are negative for AR and are androgen-independent, as we have demonstrated in a recent publication (1). We hypothesize that hormonal therapy induces differentiation of some secretory type cancer cells to NE cells and these NE cells survive hormonal therapy and are responsible for tumor recurrence. We further hypothesize that hormonal therapy causes changes in multiple signaling pathways in PC cells, and coordination and cross-talk of the different pathways lead to NE differentiation. The main objective is to study the mechanism of action of PTP1B in androgen withdrawal-induced NE differentiation, in relation to other important intracellular signaling pathways. Three tasks were proposed in the original proposal: 1). The function of PTP1B in the regulation of IL-8 signaling in PC; 2). The function of PTP1B in IGF-1 receptor signaling through PI3K/AKT/mTOR pathway; 3). The function of PTP1B in androgen receptor signaling.

Body:

1. Research accomplishments associated with task 1: The function of PTP1B in the regulation of IL-8 signaling in PC

Task 1A: Does IL-8 signaling change the expression and activity of PTP1B? To accomplish this task, we proposed to establish LNCaP cells stably expressing CXCR2 to determine if IL8-CXCR2 interaction upregulates the expression of PTP1B and whether PTP1B is required for the function of CXCR2 in promoting NE differentiation of PC

Accomplishments:

1. Establishment of stable cell lines overexpressing CXCR2: The objective of establishing such stable cell lines is to determine if activation of CXCR2 leads to increased expression of PTP1B. IL-8 has two different receptors, CXCR1 and CXCR2. In a recent publication, based on immunohistochemical study of human tissue, we found very different patterns of expression of CXCR1 and CXCR2 in different subpopulations of cancer cells of human PC (2). We hypothesize that IL-8-CXCR1 interaction stimulates PC cell proliferation while IL-8-CXCR2 interaction promotes NE differentiation of PC cells. Therefore, activation of CXCR1 and CXCR2 may have totally different consequences in PC cells. Although we did not propose to study CXCR1 in the original grant application, we felt that LNCaP cells overexpressing CXCR1 would be a good control for our research studying the function of IL-8-CXCR2 signaling. Therefore, at the time of establishing stable LNCaP cells overexpressing CXCR2, we also established

LNCaP cells overexpressing CXCR1 (Both pSFFV-CXCR1 and pSFFV-CXCR2 construct were received from Dr. Schraufstatter, La Jolla Institute for Molecular Medicine). To ensure successful transfection and protein expression, we released CXCR1 and CXCR2 cDNAs from the pSFFV vector and inserted them into the pcDNA3 vector and obtained pcDNA3-CXCR1 and pcDNA3-CXCR2 constructs. These constructs were transfected into LNCaP cells and the transfected LNCaP cells were cultured in the presence of G418. G418-resistent clones were expanded to prepare frozen stocks of the stable cell lines. We then studied the expression of CXCR1 and CXCR2 in these clones by real-time PCR. An example of the real-time PCR results is shown in Figure 1 which shows that the stable clones expressed significantly higher levels of CXCR1 and CXCR2 mRNAs, respectively, than cells transfected with vector only.

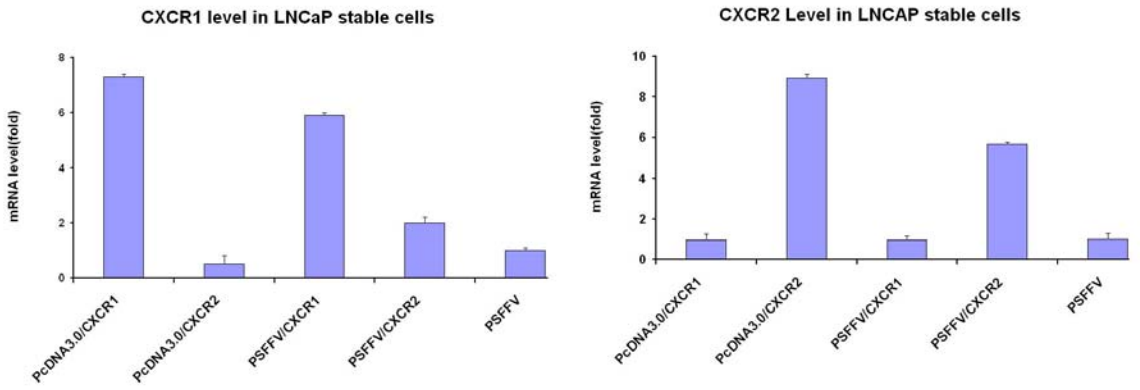

Figure 1. A real-time PCR assay shows overexpression of CXCR1 and CXCR2 in LNCaP cells stably transfected with CXCR1 and CXCR2 cDNA, respectively.

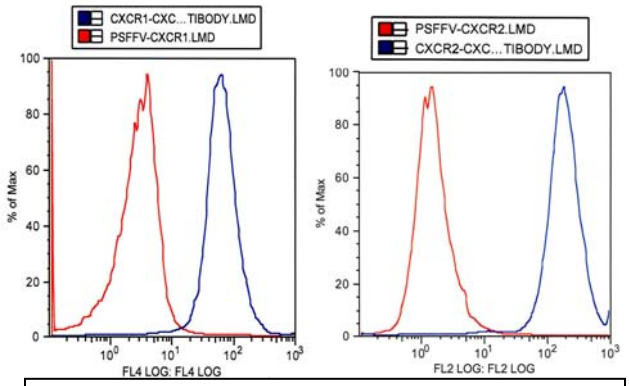

Figure 2. Flow cytometry shows ver expression of CXCR1 and CXCR2 in LNCaP cells stably transfected with CXCR1 and CXCR2 cDNA, respectively. Red: parental cells; Blue: stable clones

In order to confirm that the receptor proteins are expressed on the cell surface, we performed flow cytometry study. Figure 2 shows that, similar to the results obtained in real time PCR studies, LNCaP cells stably transfected with CXCR1 and CXCR2 vDNAs expressed higher levels of the receptors than cells transfected with vector only. Therefore, we have successfully established stable cell lines expressing CXCR1 and CXCR2, which will allow us to study if CXCR2 activation by IL-8 changes the expression of PTP1B.

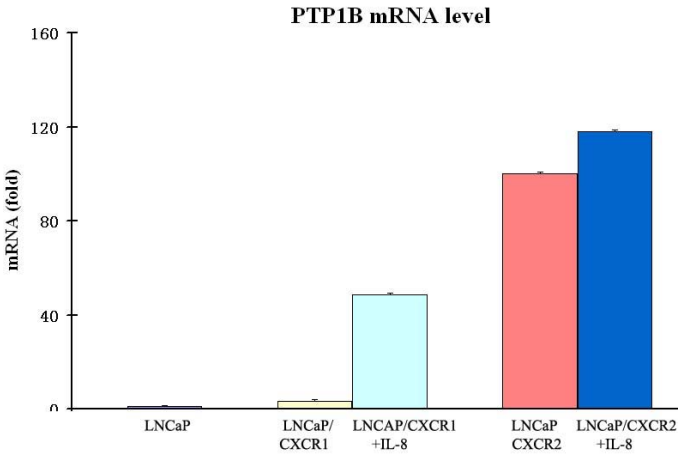

Figure 3. IL-8 mRNA levels in LNCaP cells after ectopic expression of CXCR1 and CXCR2 (Real-time PCR)

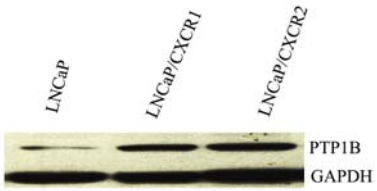

Figure 4. Western blot shows the protein level of PTP1B after ectopic expression of CXCR1 and CXCR2.

In the next set of experiments, we wanted to study if activation of the ectopically expressed CXCR2 by IL-8 changes the expression of PTP1B. Figure 3 shows that in the parental LNCaP cells, the expression of PTP1B is low, which did not significantly change with the expression of CXCR1. However, expression of CXCR2 induced overexpression of PTP1B mRNA by more than 100 fold. Interestingly, although expression of CXCR1 did not change PTP1B expression, addition of IL-8 to the cultured cells increased the expression level of PTP1B by >50 fold. Addition of IL-8 to the LNCaP-CXCR2 cells induced additional PTP1B expression (approximately 20%). These results from the real-time PCR were supported by western blot (Figure 4). These results support our original hypothesis that PTP1B may participate in the intracellular signaling of CXCR2.

Our next experiment was to determine if activation of CXCR2 in LNCaP cells induces NE differentiation. A feature of NE differentiation in LNCaP cells is the expression of NE marker neuron-specific enolase (NSE). We found that in LNCaP cells expressing either CXCR1 or CXCR2, the basal levels of NSE increased significantly, which was further increased after IL-8 stimulation for 6 days. (Figure 5, 6).

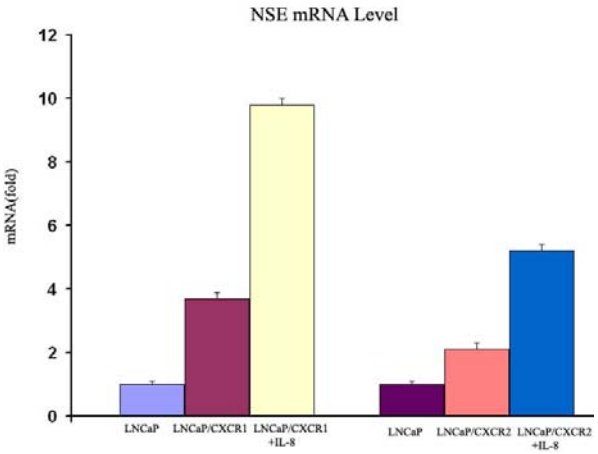

Figure 5. Real-time PCR assay shows the basal levels of NSE in LNCaP, LNCaP/CXCR1 and LNCaP/CXCR2 cells as well as levels of NSE in these cells after IL-8 stimulation for 6 days, respectively.

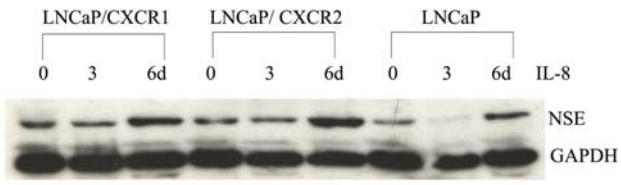

Figure 6. Western blot shows the basal levels of NSE in LNCaP, LNCaP/CXCR1 and LNCaP/CXCR2 cells as well as levels of NSE in these cells after IL-8 stimulation for 3 and 6 days, respectively.

Task 1B: Does PTP1B activate IL-8 signaling pathway in PC? To accomplish this task, we proposed to use LNCaP cells overexpressing PTP1B (LNCaP/PTP1B cells) to determine if overexpression of PTP1B upregulates the expression of IL-8 and its receptor CXCR2 and whether CXCR2 signaling is required for the function of PTP1B in promoting NE differentiation of PC

In a recent publication, we have shown that LNCaP cells overexpressing wild type PTP1B (LNCaP/PTP1B) undergo NE differentiation without any stimulation (3), suggesting that PTP1B promotes NE differentiation. The goal of this task is to determine whether NE differentiation induced by PTP1B is mediated by increased expression of IL-8 or IL-8 receptor CXCR2. To test this hypothesis, we used parental LNCaP cells, LNCaP/PTP1B cells and LNCaP cells expressing a mutant PTP1B which does not have enzymatic activity (LNCaP/PTP1BM). A real-time PCR assay showed that ectopic expression of PTP1B increases the mRNA level of IL-8 (Figure 7). This result was confirmed by an ELISA assay showing increased levels of IL-8 in the conditioned media of the LNCaP/PTP1B cells (Figure 8). It is curious that in LNCaP/PTP1BM cells, expression of IL-8 was also moderately increased.

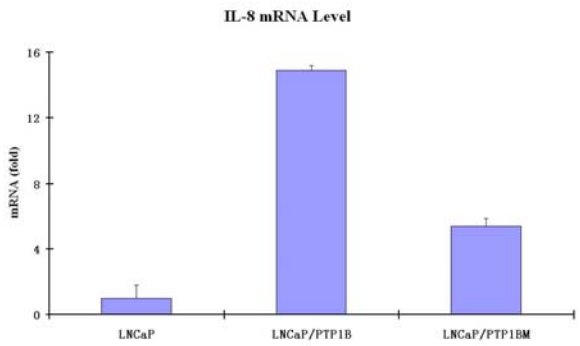

Figure 7: Real time PCR assay showing the mRNA levels of IL-8 in LNCaP cells and LNCaP cells expressing wild type PTP1B (LNCaP/PTP1B) and a mutant PTP1B (LNCaP/PTP1BM)

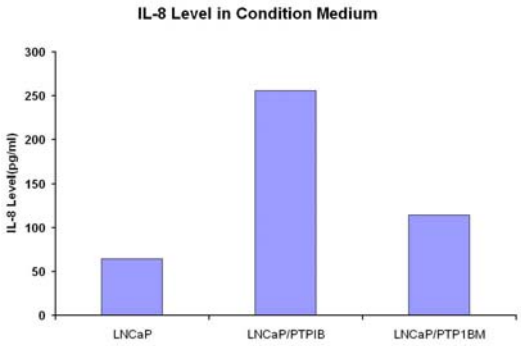

Figure 8: ELISA PCR assay showing the levels of IL-8 in conditioned media of LNCaP cells and LNCaP cells expressing wild type PTP1B (LNCaP/PTP1B) and a mutant PTP1B (LNCaP/PTP1BM)

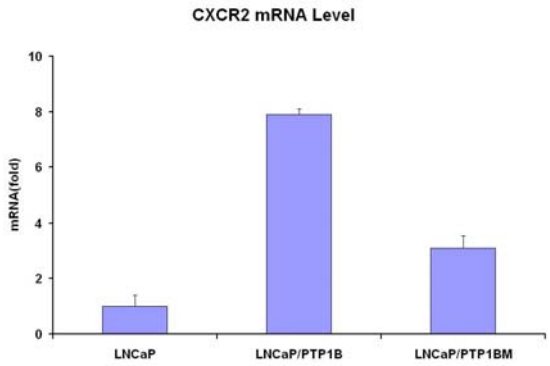

Figure 9. Real-time PCR assay shows the expression levels of of CXCR2 mRNA in LNCaP cells and LNCaP cells expressing wild type PTP1B (LNCaP/PTP1B) and a mutant PTP1B (LNCaP/PTP1BM)

The other experiment proposed in this task was to determine if PTP1B regulates the expression level of IL-8 receptor CXCR2. To test this hypothesis, we have studied the expression of CXCR2 in LNCaP/PTP1B cells in comparison to that in the parental LNCaP cells. We showed that expression of PTP1B significantly increased the level of CXCR2 mRNA and the results are shown in Figure 9.

Task 1C. Does PTP1B downregulate the expression of CXCR1? Our previous studies

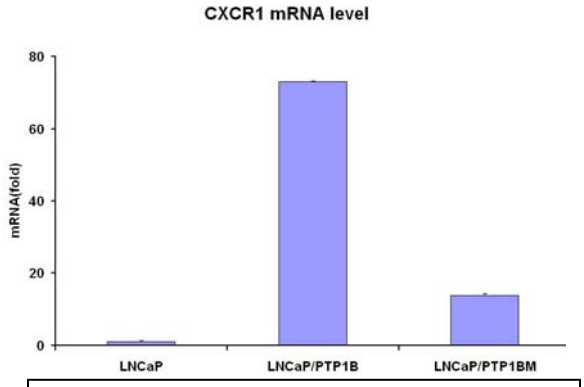

Figure 10. Real-time PCR assay shows the expression levels of of CXCR1 mRNA in LNCaP cells and LNCaP cells expressing wild type PTP1B (LNCaP/PTP1B) and a mutant PTP1B (LNCaP/PTP1BM)

have shown that proliferative activity of PC cells and NE phenotype are mutually exclusive. Since our recent publication has shown that CXCR1 and CXCR2 are expressed in non-NE tumor cells (proliferative) and NE tumor cells (non-proliferative), respectively, in a mutually exclusive fashion, we proposed to study if NE differentiation of PC cells (decreased proliferation) is associated with decreased expression of CXCR1. In order to answer this question, we proposed to use LNCaP cells overexpressing PTP1B (LNCaP/PTP1B cells) to determine if the expression of CXCR1 is decreased in such cells in comparison to the parental LNCaP cells. To the contrary, we observed a significant increase in the level of CXCR1 in LNCaP cells expressing PTP1B. The results are shown in Figure 10.

Research accomplishments associated with task 2. The function of PTP1B in IGF-1 receptor signaling through PI3K/AKT/mTOR pathway

When the proposal was being submitted, we had obtained some preliminary data showing that androgen withdrawal in LNCaP cells induces activation of the PI3 kinase-AKT-mTOR pathway, which is required for NE differentiation induced by androgen withdrawal. Since PI3K-AKT-mTOR pathway is classically activated by a cell surface protein tyrosine kinase receptor, we hypothesize that androgen withdrawal activates such a receptor, leading to activation of the PI3K-AKT-mTOR pathway, resulting in NE differentiation.

A leading candidate protein tyrosine kinase receptor is IGF-1 receptor, which has been implicated in many aspects of PC. We have shown that IGF-1 can induce PI3K-AKT-mTOR pathway and NE differentiation in LNCaP cells. A manuscript describing these important findings was accepted and published in the Journal of Biological Chemistry (4) (manuscript attached in the appendix).

Task 3. The function of PTP1B in androgen receptor signaling

Work related to this task will be performed later.

Additional significant findings that were not anticipated in the original proposal

Our ultimate goal is to determine the molecular mechanisms that are responsible for the recurrence of PC after hormonal therapy. We hypothesize that NE tumor cells, being AR negative, are resistant to hormonal therapy that targets AR signaling and will survive hormonal therapy, which may lead to tumor recurrence. As we focused our research on the function of NE cells in PC, we started paying more attention to the cancer

stem cells theory. Although cancerous cells are considered monoclonal, it has been increasingly recognized that hierarchy exists among them. It has been known for over 50 years that not all cells from a given cancer are capable of reproducing the parental tumor. In-vitro and in-vivo assays have shown that a minor population (typically 1-2%) of tumor cells shows extensive proliferation and has much higher tumorigenicity. They are considered cancer stem cells and it is hypothesized that only the cancer stem cells are capable of giving rise to new tumors (reviewed by Jordan (5)). This hierarchical cancer stem cell model predicts that therapy (e.g., hormonal therapy for PC) effective against the bulk cancer cell population but not cancer stem cells, while initially effective, would be doomed to eventual failure. Therefore, stem cells in human PC may be important therapeutic targets.

Cancer stem cells were first demonstrated in leukemia by Bonnet and Dick et al (6). Cancer stem cells have also been shown to exist in certain solid tumors, which was first demonstrated in breast cancer by Al-Hajj et al (7). Singh et al showed that the neural stem cell antigen CD133 is expressed in brain-derived cancer stem cells from pediatric medulloblastomas and astrocytomas (8). Stem cells have not been definitively identified in human PC tissue despite much effort by many research groups. During the course of our study, we started realizing that NE tumor cells in PC possess many of the characteristics described for cancer stem cells. For example, they comprise a minor component (1-2%) of the tumor cells, do not express differentiation markers such as AR and PSA, are usually quiescent and are resistant to conventional therapy.

Tang's group has published a comprehensive study in cell lines and animal models showing that CD44+ cells possess features of cancer stem cells of PC (9). However, the expression of CD44 in human PC tissue has not been examined in detail, particularly its relationship to NE tumor cells. We have since extended our research studying the relationship of CD44 expression and NE tumor cells of PC and obtained the following results:

1). CD 44 expression is associated with NE phenotype in PC cell lines

By using an antibody against all isoforms of CD44, we show that, among the three classic PC cell lines, LNCaP cells are mostly negative for CD44 (with few CD44 positive cells), DU145 cells contain both CD44- and CD44+ cells while PC3 cells are mostly positive for CD44 (with few CD44 negative cells) (Fig. 11). These results are consistent with those reported in the literature (9).

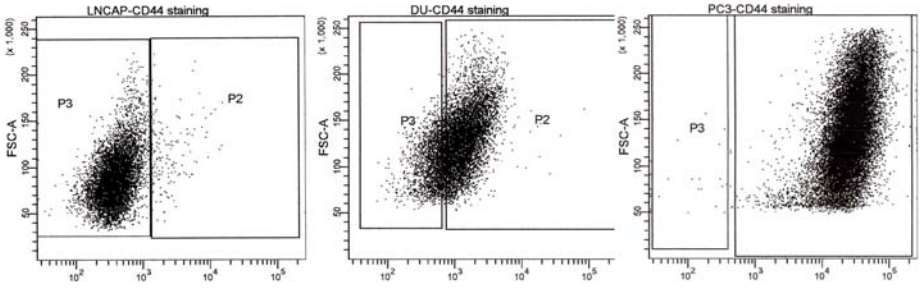

Fig. 11. Flow cytometric analysis of CD44 expression in PC cell lines. LNCaP cells (left panel) are mostly negative for CD44, DU145 cells (middle panel) contain both CD44- and CD44+ cells, while PC3 (right panel) are mostly positive for CD44 with only few CD44- cells.

We next studied the NE phenotype of the three cell lines to determine if there is a correlation between CD44 expression and NE phenotype. The most commonly used markers for NE tumor cells of PC include chromogranin A (CgA), synaptophysin and NSE. Among them, anti-CgA antibody works best for staining tissue sections while anti-

NSE antibody is the best choice for cultured cell lines (western blot and immunofluorescence). Our study showed that LNCaP cells do not express NE markers while DU145 and PC3 cells do (Fig. 12, A-C), a pattern that parallels CD44 expression in these cells. Interestingly, the NE marker and CD44-negative LNCaP cells are positive for AR and PSA while the NE-marker and CD44 positive DU145 and PC3 cells are negative for AR and PSA (Fig. 12, D-E), consistent with our published data that NE tumor cells in human PC tissue do not express AR and PSA (1).

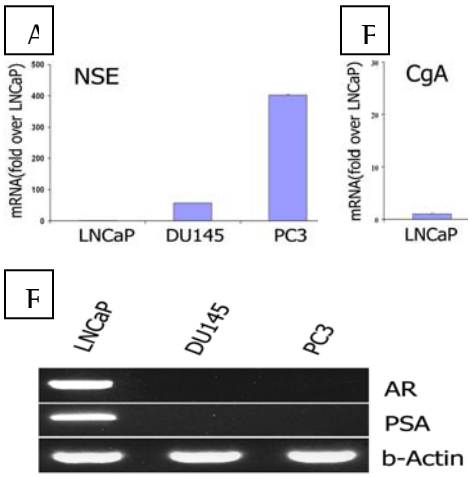

Fig. 12. Expression of NE markers, AR and PSA in PC cell lines. A. Real-time PCR showing the mRNA levels of NSE; B. Real-time PCR showing the mRNA levels of CgA; C. Western blot showing the protein levels of NSE; D. Western blot showing the protein levels of AR; E. RT-PCR showing the expression of AR and PSA.

We then flow sorted LNCaP, DU145 and PC3 cells based on CD44 expression. As shown in Fig. 11, the expression levels of CD44 vary considerably within the same cell line. Therefore, we used top 5% (CD44H) and bottom 5% of the cells (CD44L) of the cells. Real-time PCR showed that the CD44H cells had higher levels of the NE markers than the CD44L cells. The results were confirmed by western blot when enough cells were available after sorting (Fig. 13)

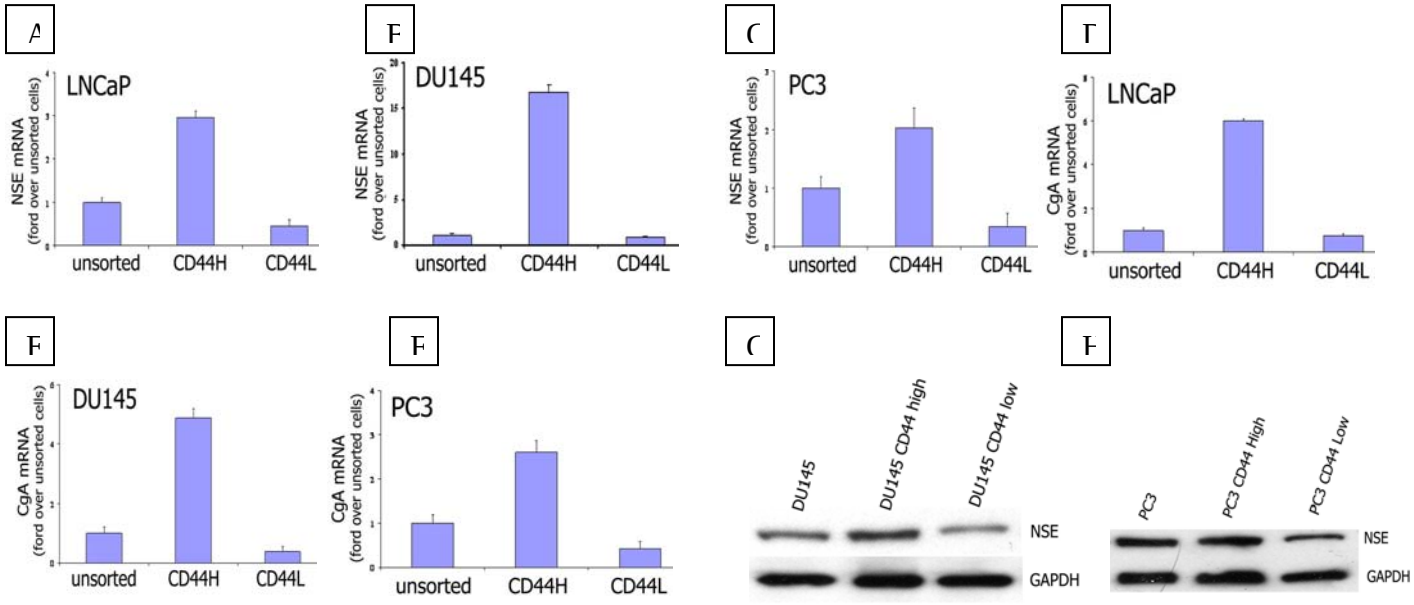

Fig. 13. CD44 high PC cells express higher levels of NE markers than CD44 low cells. A-C. Real-time PCR assay for NSE mRNA levels in LNCaP, DU145 and PC3 cells (unsorted, CD44 high, CD44 low); D-F. Real-time PCR assay for CgA levels in LNCaP, DU145 and PC3 cells (unsorted, CD44 high and CD44 low); G-H. Western blots showing the protein levels of NSE in DU145 and PC3 cells (unsorted, CD44 high and CD44 low).

Fig. 14 shows that when the PC cell lines were double-stained by immunofluorescence for CD44 and NE marker NSE, LNCaP cells were essentially negative for both, PC3 cells were mostly positive for both while DU145 cells contained both positive and negative populations. Importantly, CD44 positive cells were positive for NSE and vice versa, confirming that the CD44+ cells were the NE tumor cells.

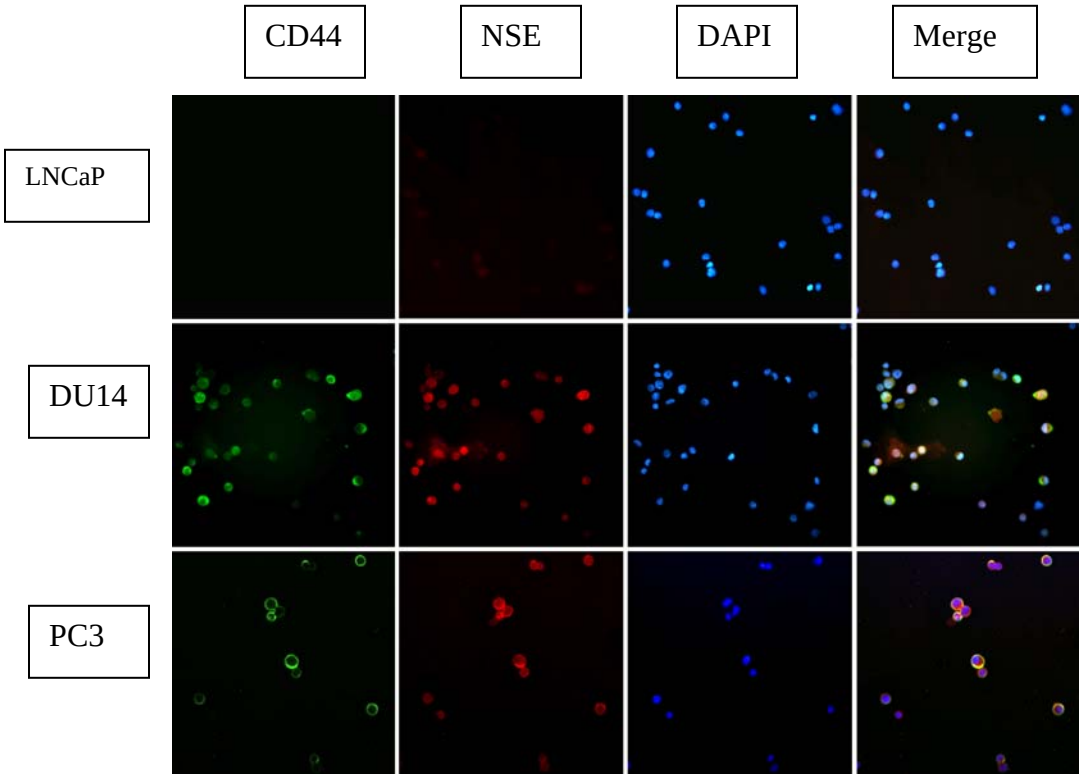

Fig 14. Immunoflurescence staining for the co-expression of CD44 and NSE. LNCaP cells are essentially negative for both markers, PC3 cells are mostly positive for both markers, while DU145 contain both positive and negative populations. CD44 positive cells are always positive for NE marker NSE, suggesting that they are NE cells. (color figure)

2). NE cell phenotype is associated with CD44+ cells in single cell suspension of fresh human PC tissue

The above results strongly suggest that in PC cell lines, NE phenotype is associated with the expression of CD44, which has been shown to be a marker that identifies cancer stem/progenitor cells of PC (9,10). In order to show the relevance of this finding to human disease, we studied the expression of CD44 in NE tumor cells from fresh tumor tissue of PC patients. Patients consented to the study before surgery. As soon as the prostates were removed, they were brought to surgical pathology for gross examination. Excess tumor tissue not required for pathologic diagnosis was used to prepare single cell suspensions. The cells were then spun onto charged slides by cytospin for immunofluorescence study to determine if expression of CD44 was limited to NE tumor cells. Approximately 1-2% of human tumor cells were positive for CD44, consistent with the estimated number of stem/progenitor cells in human PC, which is also the approximate number of NE tumor cells seen in PC. Similar to PC cell lines, CD44+ human PC cells were positive for CgA (Fig. 15), confirming that NE tumor cells are the CD44+ cells, and thus may represent the stem/progenitor cells in human PC.

We subjected single cell suspensions from 7 cases of fresh human PC to flow-sorting to separate CD44-high and CD44-low cells. Because the CD44-high cells were few, there was only enough material for quantitative real-time PCR. In every single case, the CD44 positive cells always expressed higher levels of NE marker CgA and NSE than

the CD44 negative cells (Fig. 16), consistent with the former being NE tumor cells of PC. We did notice that the differences of NE marker levels varied from case to case, which was probably due to some contamination by other CD44+ cells (such as basal cells in benign prostate and lymphocytes, see later).

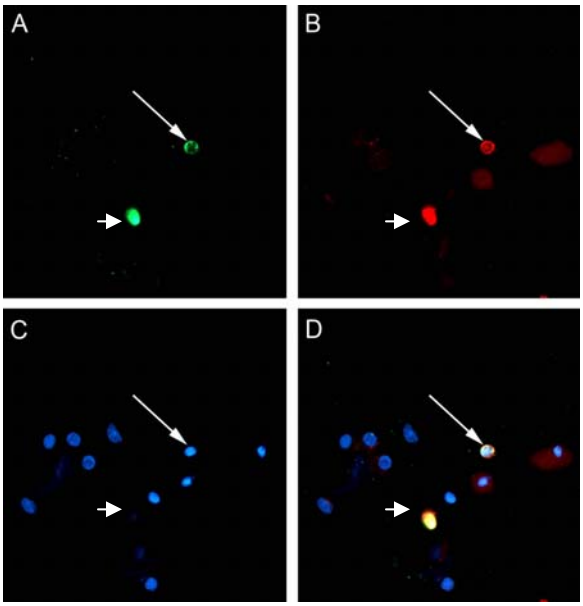

Fig. 15. Immunofluorescence study showing co-expression of CD44 with NE cell marker CgA in single cells suspension from fresh human PC. A. CD44; B. CgA; C. Hoechst 33258. D. Merged picture. The single CD44+ NE tumor cell in the field is marked by a long arrow. The other brightly-stained spot is a contaminant since it is not associated with a nucleus (short arrow).(Color figure)

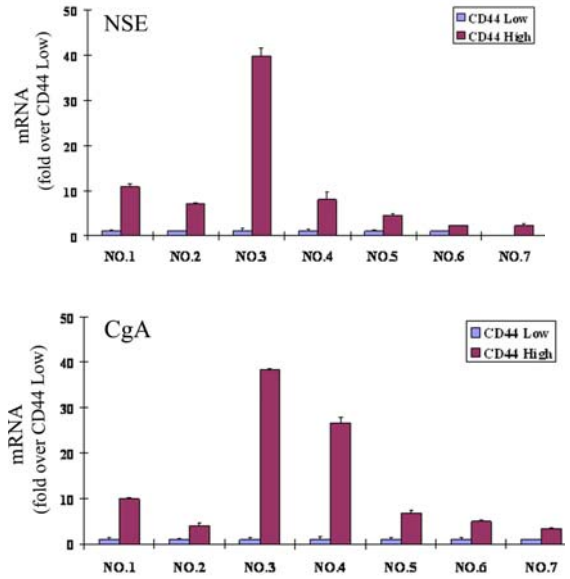

Fig. 16. Single cell suspension from 7 cases of fresh PC tissue was subjected to flow sorting. Quantitative real-time PCR was performed on CD44 low (blue columns) and CD44 high (red columns) cells for the expression of NE markers NSE (upper panel) and CgA (lower panel). (color figure)

3). Expression of CD44 in NE cells of PC tissue

Results of the previous experiments performed with PC cell lines and single cell suspension of fresh human tumors strongly suggest that the NE cells in PC are the CD44+ PC cells which have been demonstrated to possess cancer stem/progenitor cell features (9). Traditionally, studies on NE cells of PC have been mostly carried out in human PC tissue sections by immunohistochemistry, which is responsible for much of our current knowledge about these cells. Therefore, we performed additional studies to confirm these findings in human PC tissue sections.

We have accumulated significant experience and expertise in studying NE cells (1-4). We have constructed tissue microarrays (TMA) containing hundreds of cases of PC. Immunohistochemical study shows that in benign prostate, the basal cells express CD44, as has been reported (11). Nerves and lymphocytes, which can be present in benign prostate and PC, also express CD44. The majority of the tumor cells in PC are entirely negative for CD44. However, there are scattered individual tumor cells or small nests of tumor cells that stained strongly for CD44 (Fig. 17). The morphology and distribution of these CD44+ tumor cells in PC were reminiscent of NE tumor cells. In

order to prove that these scattered CD44+ tumor cells are NE tumor cells, we stained adjacent sections (which contained nearly identical tumor cells) with an anti-CD44 and an anti-CgA antibody, respectively. When the two sections were compared, it was clear that the CD44+ cells in PC were the CgA+ NE tumor cells (Fig. 18).

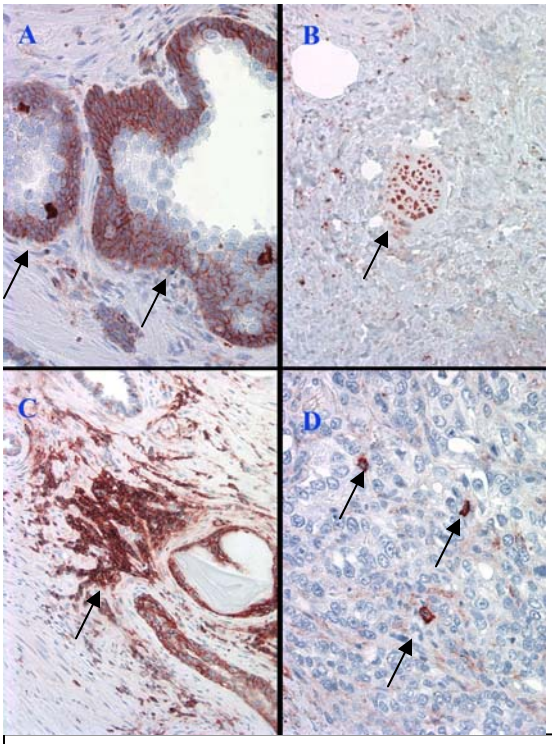

Fig. 17. CD44 is expressed in basal cells of benign prostate (A, arrows), nerve (B, arrow), lymphocytes (C, arrow) and scattered tumor cells in PC (D, arrows) (color figure)

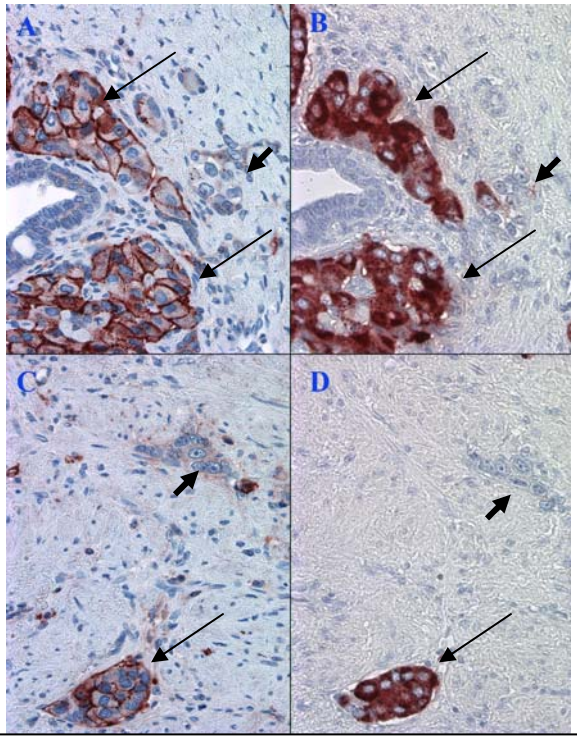

Fig 18. The first sections (A,C) of PC tissue were stained for CD44 and the adjacent sections (B,D) were stained for CgA to highlight the NE tumor cells. CD44+ tumor cells (arrows in A,C) are NE tumor cells (arrows in B,D). CD44 negative tumor cells (arrowheads in A,C) are non-NE tumor cells (arrowheads in B,D) (color figure)

We further confirmed this finding with an immunofluorescence study by staining the same TMA section containing PCs with both anti-CD44 and anti-CgA antibodies. Similarly, expression of CD44 is limited to the NE tumor cells (Fig 19).

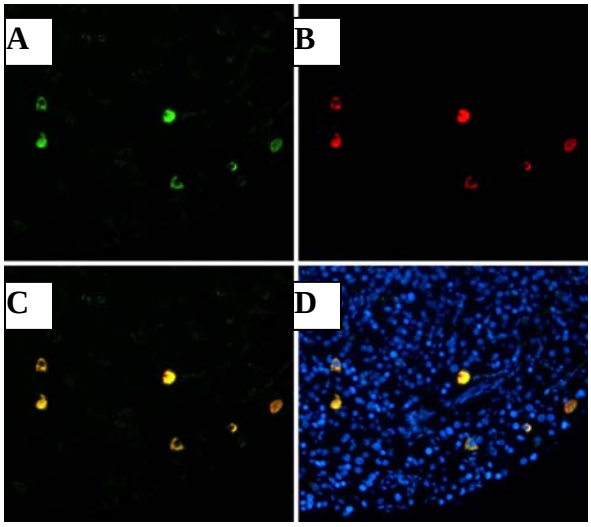

Fig. 19. Immunofluorescence study shows co-expression of CD44 and CgA in the same tumor cells. A. CD44; B. CgA; C. CD44/CgA merge; D. Merged picture with DAPI showing that the NE tumor cells are CD44 positive and comprise approximately 1% of the total tumor cells in PC..(color figure)

Therefore, we have unequivocally proven in human PC tissue that NE tumor cells are the CD44+ cells. We have previously reported that NE tumor cells are quiescent and

do not express AR and PSA (1). Our results, in combination with the report by Patrawala et al (9) showing that CD44 expression identifies cancer stem cells, strongly suggest that NE tumor cells of PC may represent the PC stem cells.

The above results have been submitted to the upcoming 2008 AACR meeting in San Diego (see appended abstract).

3). Expression of CD44 in small cell carcinomas of PC

NE cells in PC are usually quiescent and do not express AR and PSA, consistent with them being cancer stem cells. A feature of cancer stem cells is that although they are normally quiescent, they also possess unlimited proliferative potential under appropriate stimulation. The majority of the PCs are adenocarcinomas but a small minority of the cancers are the so-called small cell carcinomas which are composed entirely of NE tumor cells that are highly proliferative. Similarly the tumor cells do not express AR and PSA. We hypothesize that the tumor cells in adenocarcinoma cells are differentiated tumor cells but in small cell carcinomas, the tumor cells remain undifferentiated and possess the stem cell features. Should that be the case, the tumor cells should express stem cell-associated marker CD44. We stained 12 cases of small cell carcinomas of the prostate and 11 cases showed diffuse and strong membrane staining (Figure 20) while the majority of the small cell carcinomas from other organs were entirely negative for this marker. An abstract describing this finding has been accepted for presentation at the Annual Meeting of the United States and Canadian Academy of Pathology to be held in Denver in March 2008 (see appended abstract).

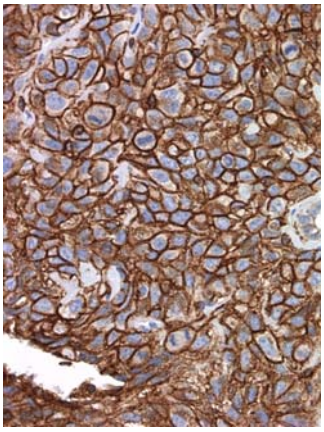

Figure 20. Immunohistochemical staining shows strong and diffuse membrane staining for CD44 in a case of small cell carcinoma of the prostate

Key research accomplishments:

1. We have established LNCaP cell lines stably expressing CXCR1 and CXCR2.
2. We found that in LNCaP cell lines stably expressing CXCR1 and CXCR2, PTP1B is overexpressed when compared with parental cells.
3. In LNCaP cell lines stably expressing CXCR1 and CXCR2, there is also significant increase in the expression of NSE, a marker of neuroendocrine differentiation.
4. We studied the expression of IL-8 in LNCaP cells stably overexpressing PTP1B and a mutant PTP1B and found increased levels of IL-8 in such cell lines in comparison to parental LNCaP cells.
5. Expression of IL-8 receptors CXCR1 and CXCR2 are also increased in LNCaP cells stably overexpressing PTP1B and a mutant PTP1B
6. We have accumulated evidence that the neuroendocrine cells may be the stem cells of prostate cancer.

Reportable outcomes:

Manuscript:
Wu C, **Huang J**. Phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin pathway is essential for neuroendocrine differentiation of prostate cancer. **The Journal of Biological Chemistry** 2007;282(6):3571-3583

Abstracts:
1. Guangchun Chen, Christopher R. Silvers, Chengyu Wu, Linda Salamone, Heather B. Martin, Karin Williams, Ganesh S. Palapattu, **Jiaoti Huang**. Neuroendocrine-like cells in prostate cancer express CD44 and may represent the putative cancer stem/progenitor cells. Submitted to the AACR annual meeting 2008

2. RA Simon, H Xu, PA Bourne, JL Yao, J Wei, S Liang, J Liu, PA di Sant'Agnese, L Cheng and **J Huang**. CD44 is a Useful Marker for Prostatic Small Cell Carcinoma. 2008 United States and Canadian Academy of Pathology Annual Meeting

Stable cell lines established

1. LNCaP cells overexpressing CXCR1
2. LNCaP cells overexpressing CXCR2

Conclusion:

Neuroendocrine cells, although comprising a small population of the prostate cancer cells, may be responsible for tumor recurrence after hormonal therapy as they do not express androgen receptor and are resistant to therapy. Our work demonstrates that there is a complex network of multiple signaling pathways that maintains the unique phenotype of the neuroendocrine cells, and coordination and cross-talk of the different pathways may be essential. Furthermore, we demonstrated that the neuroendocrine cells may represent the cancer stem cells of prostate cancer.

Evaluation of the knowledge as a scientific or medical product: Prostate cancer, in its localized form, can be effectively treated by surgery or radiation therapy. The currently adopted method to treat advanced and metastatic cancer is hormonal therapy. The therapy is effective initially but fails eventually in every single patient. We have demonstrated that neuroendocrine cells, although comprising a small population of the cancer, may be the cancer stem cells and resistant to hormonal therapy. Therefore, to achieve a cure, neuroendocrine cells should be targeted. We have also demonstrated complex signaling networks in maintaining the neuroendocrine phenotype of the tumor cells which are potential therapeutic targets.

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Phosphatidylinositol 3-Kinase-AKT-Mammalian Target of Rapamycin Pathway Is Essential for Neuroendocrine Differentiation of Prostate Cancer*

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Hormonal therapy of prostate cancer, by inhibiting androgen production and/or androgen function, is the treatment of choice for advanced prostate cancer. Although most patients respond initially, the effect is only temporary, and the tumor cells will resume proliferation in an androgen-deprived environment. The mechanism for androgen-independent proliferation of cancer cells is unclear. Hormonal therapy induces neuroendocrine differentiation of prostate cancer cells, which is hypothesized to contribute to tumor recurrence by a paracrine mechanism. We studied signal transduction pathways of neuroendocrine differentiation in LNCaP cells after androgen withdrawal, and we showed that both the phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin pathway and ERK are activated, but only the former is required for neuroendocrine differentiation. A constitutively active AKT promotes neuroendocrine differentiation and a dominant negative AKT inhibits it. Activation of AKT by IGF-1 leads to neuroendocrine differentiation, and neuroendocrine differentiation induced by epinephrine requires AKT activation. We also show that the AKT pathway is likely responsible for neuroendocrine differentiation in DU145, an androgen-independent prostate cancer cell line. Therefore, our study demonstrated a novel function of the AKT pathway in prostate cancer progression and identified potential targets that may be explored for the treatment of androgen-independent cancer.

Prostate cancer (PC)[2] is the most common malignancy among men in western countries (1). There are multiple treatment options for PC in early stages. For advanced and metastatic PC, hormonal therapy, consisting of androgen ablation and/or inhibition of androgen action by anti-androgen, is the treatment of choice (2). Although an initial response is seen in most patients receiving hormonal therapy, the effect is temporary, and the tumor eventually recurs and enters the androgen-independent (AI) stage in which the tumor cells proliferate in an androgen-deprived environment. There are no effective therapies for AI PC (3).

The mechanism of AI proliferation of PC is poorly understood, and many hypotheses have been proposed, such as androgen receptor (AR) amplification (4), AR mutation (3), aberrant activation of AR (5), or increased AR sensitivity to low levels of androgen in the prostate (6, 7). In addition, many studies have shown that neuroendocrine (NE) differentiation (NED) may contribute to AI growth of PC (8–10).

The epithelial compartment of benign prostate consists of luminal secretory cells, basal cells, and a minor component of NE cells that have neuron-like morphology and secret biogenic amines and neuropeptides (11). NE cells are also present in PC as scattered individual cells or small nests among the more abundant secretory type cancer cells. The number of NE cells increases in high grade and high stage tumors and particularly in hormonally treated and AI tumors (9). It is hypothesized that hormonal therapy induces NED and the NE cells contribute to AI growth of PC in the androgen-deprived environment by secreting their products to act on the adjacent non-NE tumor cells in a paracrine fashion (8–10).

Androgen withdrawal of the culture media leads to NED of LNCaP cells, a PC cell line (13), mimicking *in vivo* observation in PC patients treated hormonally. This finding supports the trans-differentiation model and suggests that NE cells may be derived from the non-NE secretory-type cancer cells (13, 14). However, the signaling pathway involved in the differentiation process is unclear. Here we report our study demonstrating that the PI3K-AKT-mTOR pathway is critically involved in NED.

EXPERIMENTAL PROCEDURES

Materials—LNCaP and DU145 cells were obtained from the American Type Culture Collection (Manassas, VA); FBS, RPMI medium 1640, sodium pyruvate, penicillin, and streptomycin were purchased from Invitrogen; charcoal/dextran-treated FBS was purchased from Hyclone (Logan, UT); GeneJuice® transfection reagent was from Novagen; RNeasy® mini kit was from Qiagen (Valencia, CA); transcriptor reverse transcriptase and homogeneous protein A were from Roche Applied Science; random hexamers was from Promega (Madison, WI); iQ™ SYBR® Green Supermix and Bio-Rad Protein assay kit were from Bio-Rad; monoclonal anti-NSE antibody was from DAKO (Carpinteria,

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[2] The abbreviations used are: PC, prostate cancer; AI, androgen-independent; NE, neuroendocrine; NED, neuroendocrine differentiation; AR, androgen receptor; S6K, S6 kinase; IGF-1, insulin-like growth factor-1; mTOR, mammalian target of rapamycin; FBS, fetal bovine serum; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; NSE, neuron-specific enolase; PI3K, phosphatidylinositol 3-kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HB-EGF, heparin-binding epidermal growth factor; C-FBS, charcoal-treated (androgen-deprived) FBS.

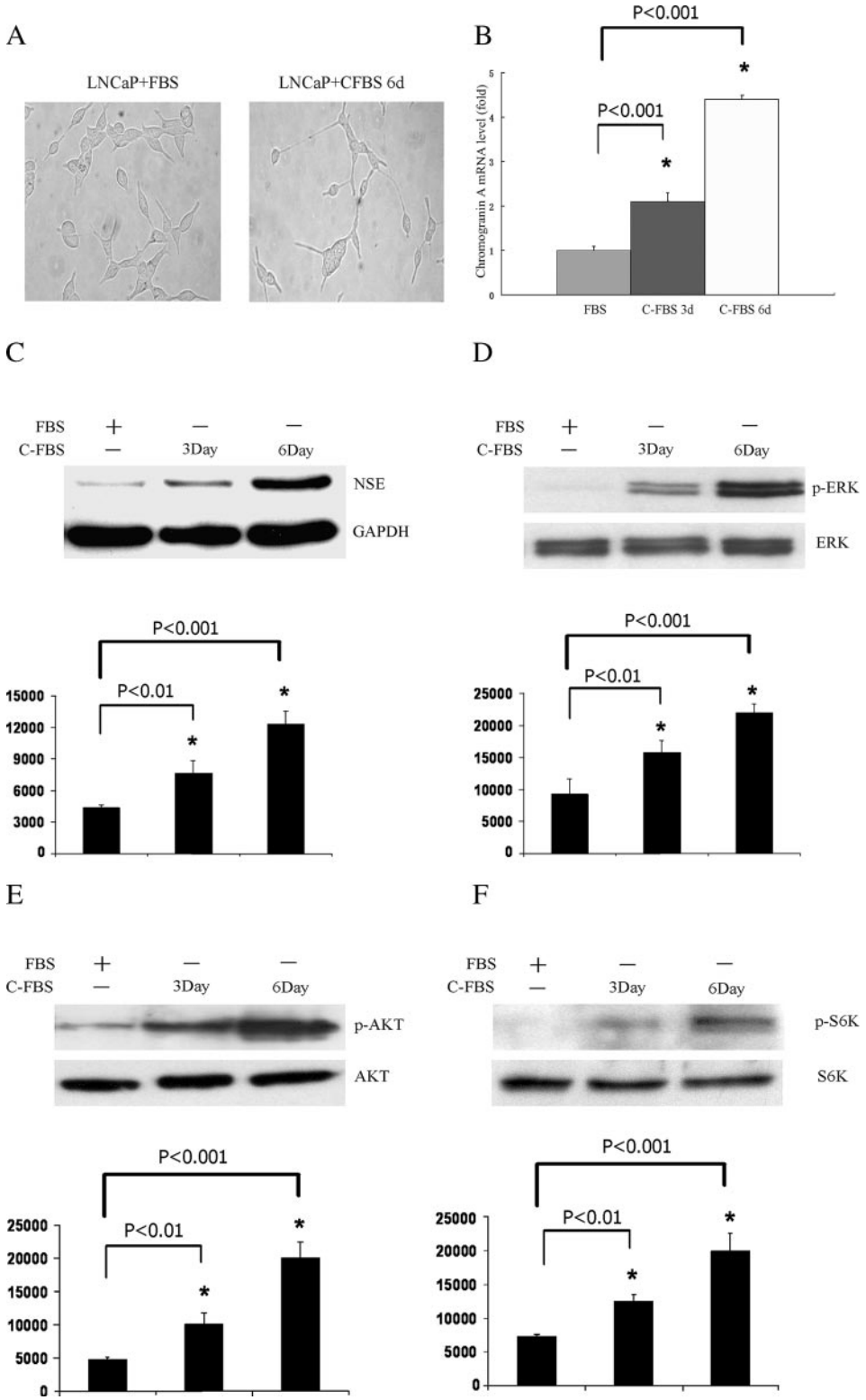


FIGURE 1. **Androgen withdrawal of cultured LNCaP cells leads to NED and activation of ERK and AKT pathways.** *A,* in comparison to LNCaP cell cultured in normal FBS, which show epithelial morphology (*left panel*), LNCaP cells cultured in charcoal-treated (androgen-deprived) FBS (*c-FBS*) for 6 days (*d*) show elongated cellular processes typical of NE cells. *B,* LNCaP cells were cultured in FBS or C-FBS for 3 and 6 days. A real time PCR assay was performed to study the expression of chromogranin A (an NE marker) mRNA. *C,* LNCaP cells were cultured in FBS or C-FBS for 3 and 6 days. Equal amounts of proteins were resolved by SDS-PAGE and immunoblotted with anti-NSE (an NE marker) and anti-GAPDH antibodies (loading control). Androgen withdrawal led to NED of LNCaP cells as characterized by increased expression of NSE. *D–F,* Western blots show increased phosphorylation of ERK, AKT, and S6K after androgen withdrawal. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.

CA); polyclonal anti-IGF-1 receptor β antibody and monoclonal anti-GAPDH antibody were from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal anti-AKT, anti-phospho-AKT, anti-ERK, anti-phospho-ERK, anti-S6K, anti-phospho-S6K antibodies, and monoclonal anti-Tyr(P)-100 antibody were from Cell Signaling (Dancers, MA); RIPA lysis buffer was from Upstate Cell Signaling Solutions (Lake Placid, NY); protease inhibitor mixture and epinephrine (used at 5 μM) were from Sigma; IGF-1 (used at 100 ng/ml) was from R&D Systems (Minneapolis, MN). U0126 (used at 10 μM) and AKT inhibitor IV (used at 20 μM) were from Calbiochem. LY294002 (used at 20 μM) was from Cayman Chemical (Ann Arbor, MI), and rapamycin (used at 10 nM) was from Biomol (Plymouth Meeting, PA).

Cell Culture, Plasmid DNA, and Transfection—LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% FBS. For androgen deprivation, cells were cultured in RPMI 1640 medium with 10% charcoal/dextran-treated FBS. When chemical inhibitors or stimulating agents (IGF-1, epinephrine) were used, the culture media were changed daily with fresh inhibitors.

pCDNA3-cAkt (a constitutively active Akt with a deletion at amino acids 4–129 replaced with a consensus myristoylation domain) (15) and pcDNA-dnAKT (kinase-deficient mutant, K179A) were kindly provided by Dr. Freeman of the University of Rochester. The liposome-mediated plasmid transfection was performed using GeneJuice® transfection reagent (Novagen). The cells were plated and maintained to 70–80% density in 50-mm plates and then transfected with the plasmid DNAs according to the protocol suggested by the manufacturer. For transient transfection, the cells were harvested 48 h after transfection. For stable transfection, the transfected cells were diluted at 1:5–1:10 and selected with 300 μg/ml G418.

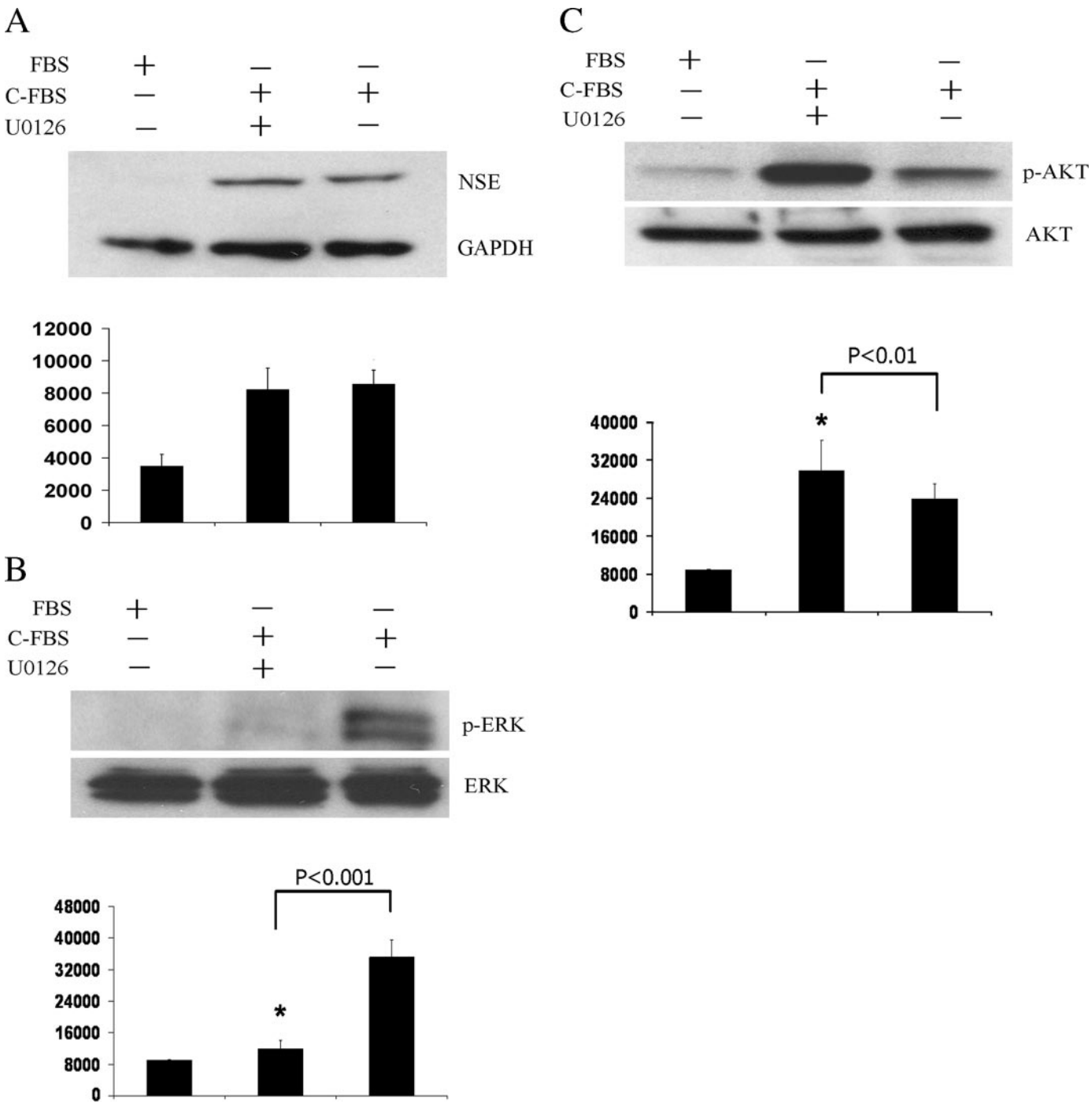


FIGURE 2. **NED induced by androgen withdrawal is not suppressed by inhibition of ERK.** *A,* LNCaP cells were cultured in FBS or C-FBS for 6 days in the absence or presence of the MEK inhibitor U0126. Equal amounts of cellular proteins were immunoblotted with anti-NSE and anti-GAPDH antibodies to show that U0126 did not inhibit NED. *B* and *C,* Western blots show that U0126 inhibited phosphorylation of ERK but increased the phosphorylation of AKT. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.

Real Time Reverse Transcription-PCR—Total RNA was isolated from cells with the RNeasy® kit. RNA was reverse-transcribed by transcriptor reverse transcriptase with random hexamers. The following specific forward and reverse primers were used for NSE, 5′-AGCTGCCCCTGCCTTAC-3′ and 5′-GAGACAAACAGCGTTACTTAG-3′, and for chromogranin A, 5′-GCGGTGGAAGAGCCATCAT-3′ and 5′-TCTGTGGCTTCACCACTTTTCTC-3′.

Real time PCR was performed with iQ™ SYBR® Green Supermix in an iCycler iQ System (Bio-Rad) using the SYBR Green Detection protocol. Total reaction volume was 20 μl, and a cycle consists of 95 °C for 5 min, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, for a total of 45 cycles followed by 72 °C for 5 min.

Western Blotting—Cells were washed twice with cold phosphate-buffered saline and lysed in RIPA lysis buffer for 30 min on ice. The cells were sheared twice through a

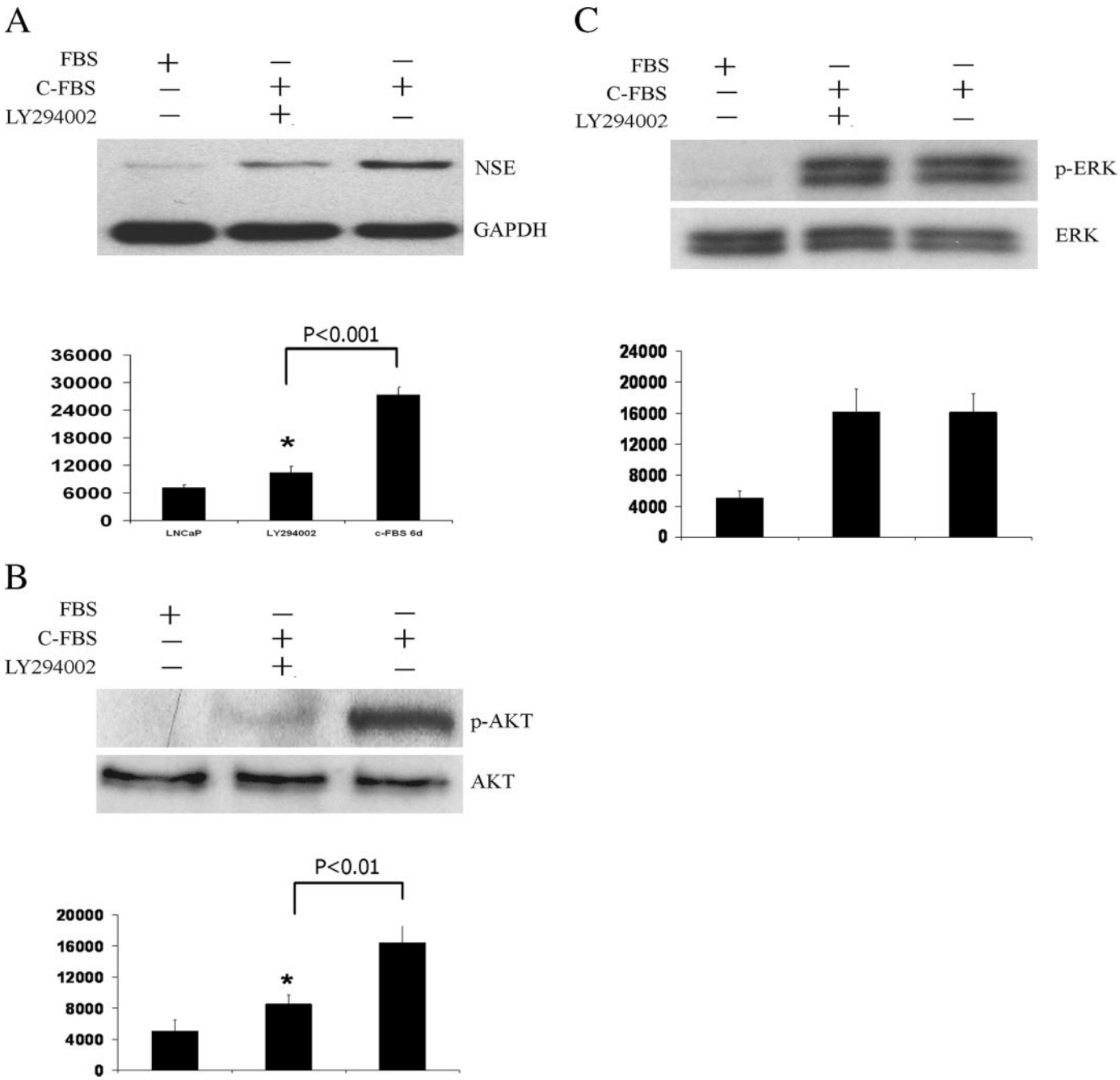


FIGURE 3. **NED induced by androgen withdrawal is suppressed by inhibition of PI3K activity.** *A–C,* LNCaP cells were cultured in FBS or C-FBS for 6 days in the absence or presence of the PI3K inhibitor LY294002. Equal amounts of cellular proteins were immunoblotted with anti-NSE, anti-GAPDH, anti-AKT, anti-phospho-AKT, anti-ERK, and anti-phospho-ERK antibodies. LY294002 inhibited NED and the phosphorylation of AKT, but not that of ERK. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.

20-gauge needle and centrifuged at 14,000 rpm for 15 min at 4 °C. The protein concentration in the supernatant was determined with the Bio-Rad protein assay kit. Equal amounts of protein were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane with Semi-Dry Transfer Cell (Bio-Rad). The membrane was blocked with TBS containing 5% w/v nonfat dry milk, hybridized with primary antibody in 2% w/v nonfat dry milk, followed by incubation with secondary antibody and detected with an ECL kit (Bio-Rad).

Statistical Analysis—Statistical significance was determined by *t* test. The results are expressed as mean ± S.D. from three separate (replicate) experiments.

RESULTS

Androgen Deprivation of LNCaP Cells Induces NED and the Activation of ERK and the PI3K-AKT-mTOR Signaling Pathways—As has been shown previously, androgen withdrawal in the culture media of LNCaP cells induced NED, characterized by changes in cell morphology (elongated cellular

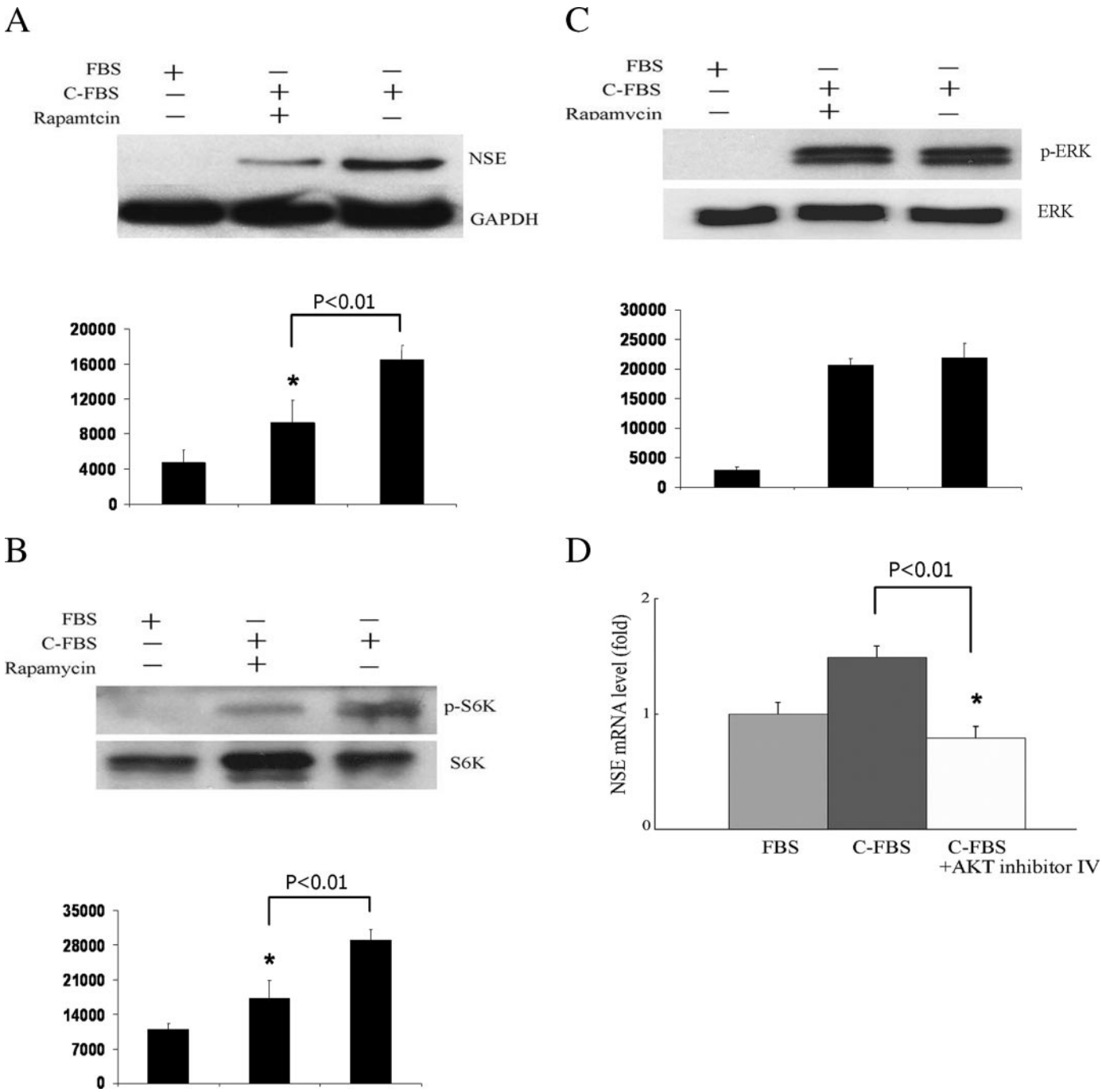


FIGURE 4. **NED induced by androgen withdrawal is suppressed by inhibition of mTOR or AKT.** *A–C,* LNCaP cells were cultured in FBS or C-FBS for 6 days in the absence or presence of the mTOR inhibitor rapamycin. Equal amounts of cellular proteins were resolved by SDS-PAGE and immunoblotted with anti-NSE, anti-GAPDH, anti-S6K, anti-phospho-S6K, anti-ERK, and anti-phospho-ERK antibodies. Rapamycin inhibited NED and the phosphorylation of S6K, but not that of ERK. *D,* LNCaP cells were cultured in FBS, C-FBS, or C-FBS plus AKT inhibitor IV for 12 h, and a real time PCR assay was performed to measure the mRNA levels of NSE in the cells. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.

processes; Fig. 1*A*) and increased expression of NE cell markers chromogranin A (Fig. 1*B*) and NSE (13) (Fig. 1*C*). Androgen withdrawal also induced phosphorylation of ERK (Fig. 1*D*) and the serine/threonine kinase AKT (Fig. 1*E*). Because AKT is a key player in the PI3K-AKT-mTOR signaling pathway and its activity is increased by phosphorylation, our observation suggests that the PI3K-AKT-mTOR pathway may be activated during NED of LNCaP cells. Consistent with this hypothesis, androgen deprivation of LNCaP cells significantly increased the levels of phosphorylation of S6 kinase (S6K) (Fig. 1*F*), an important downstream effector of mTOR whose level of phosphorylation directly correlates with the activity of the PI3K-AKT-mTOR pathway in PC (16).

The PI3K-AKT-mTOR Pathway, Not ERK Pathway, Is Required for NED of PC Induced by Androgen Withdrawal—Because androgen withdrawal activates both ERK and PI3K-AKT-mTOR pathways in LNCaP cells, we studied whether one or both of them may be required for NED induced by androgen

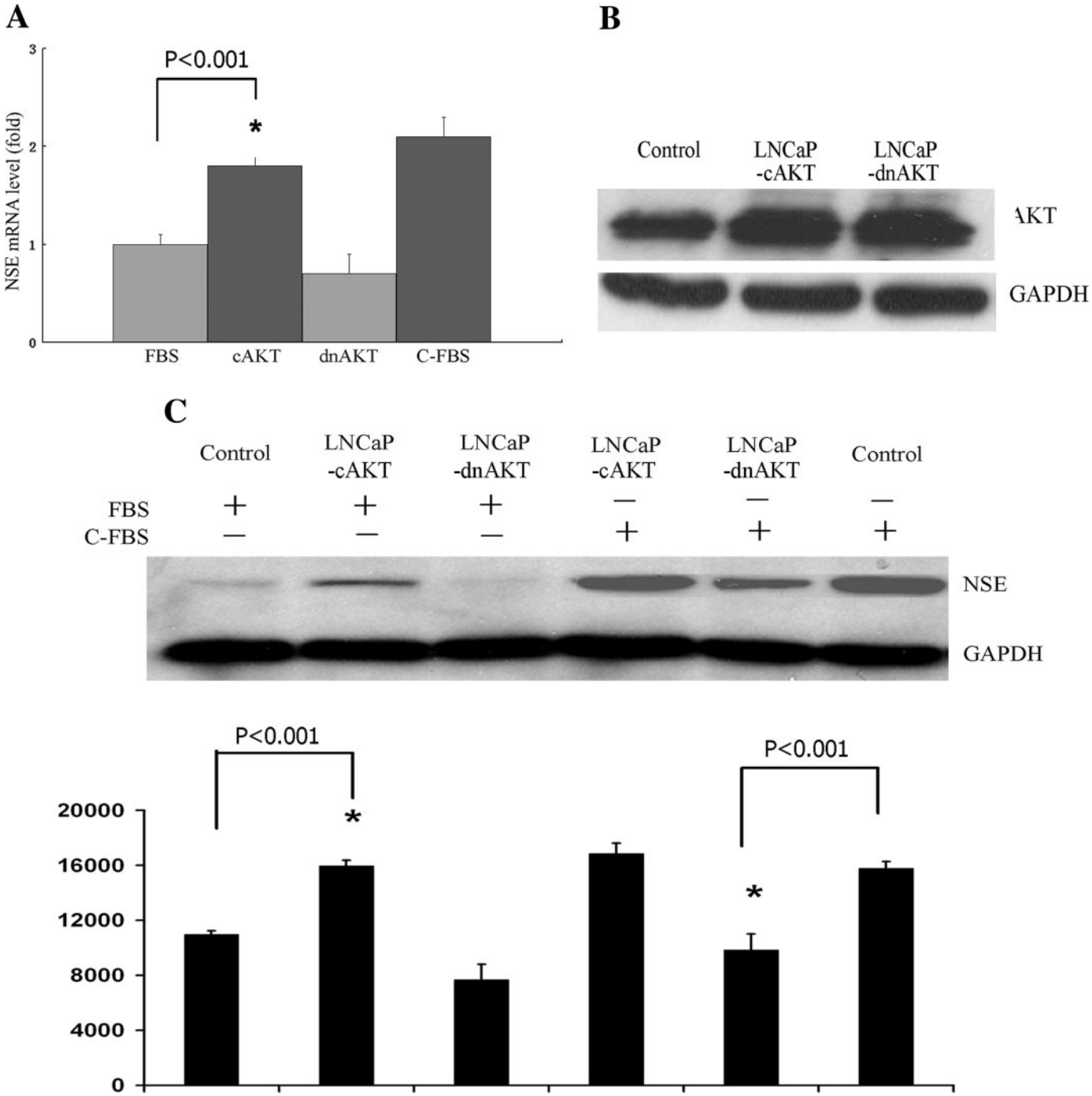


FIGURE 5. **A constitutively active AKT promotes NED while a dominant negative AKT inhibits androgen withdrawal-induced NED.** *A,* LNCaP cells were transiently transfected with pcDNA3-cAKT (expressing a constitutively active AKT) or pcDNA3-dnAKT (expressing a dominant negative AKT) or cultured in c-FBS (positive control of NED). The cells were harvested 48 h after transfection, and a real time PCR assay was performed to study the expression of NSE mRNA and showed that cAKT, not dnAKT, induced NED. *B,* equal amounts of protein from control LNCaP (transfected with empty vector), LNCaP-cAKT, and LNCaP-dnAKT cells were immunoblotted with anti-AKT antibody and anti-GAPDH antibodies. *C* and *D,* LNCaP, LNCaP-cAKT, and LNCaP-dnAKT cells were cultured in FBS or in C-FBS for 6 days. Equal amounts of proteins were immunoblotted with anti-NSE, anti-GAPDH, anti-S6K, and anti-phospho-S6K antibodies to show that cAKT induced NED and dnAKT inhibited androgen withdrawal-induced NED. *E,* LNCaP or LNCaP-cAKT cells were cultured in FBS or C-FBS for 6 days in the absence or presence of rapamycin. Equal amounts of proteins were immunoblotted with anti-NSE and anti-GAPDH antibodies to show that cAKT-induced NED was inhibited by rapamycin. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.

withdrawal. Addition of U0126, an inhibitor of MEK that inhibits the activation of ERK, had no effect on the increased NSE level in androgen-deprived LNCaP cells (Fig. 2*A*). The relative specificity of this compound was demonstrated by showing that U0126 blocked the phosphorylation of ERK (Fig. 2*B*) but not that of AKT (Fig. 2*C*), suggesting that activation of ERK is not required for NED of PC.

To study whether PI3K-AKT-mTOR pathway is required for NED of LNCaP cells, inhibitors that target key molecules of this pathway were added to LNCaP cells cultured in androgen-de-

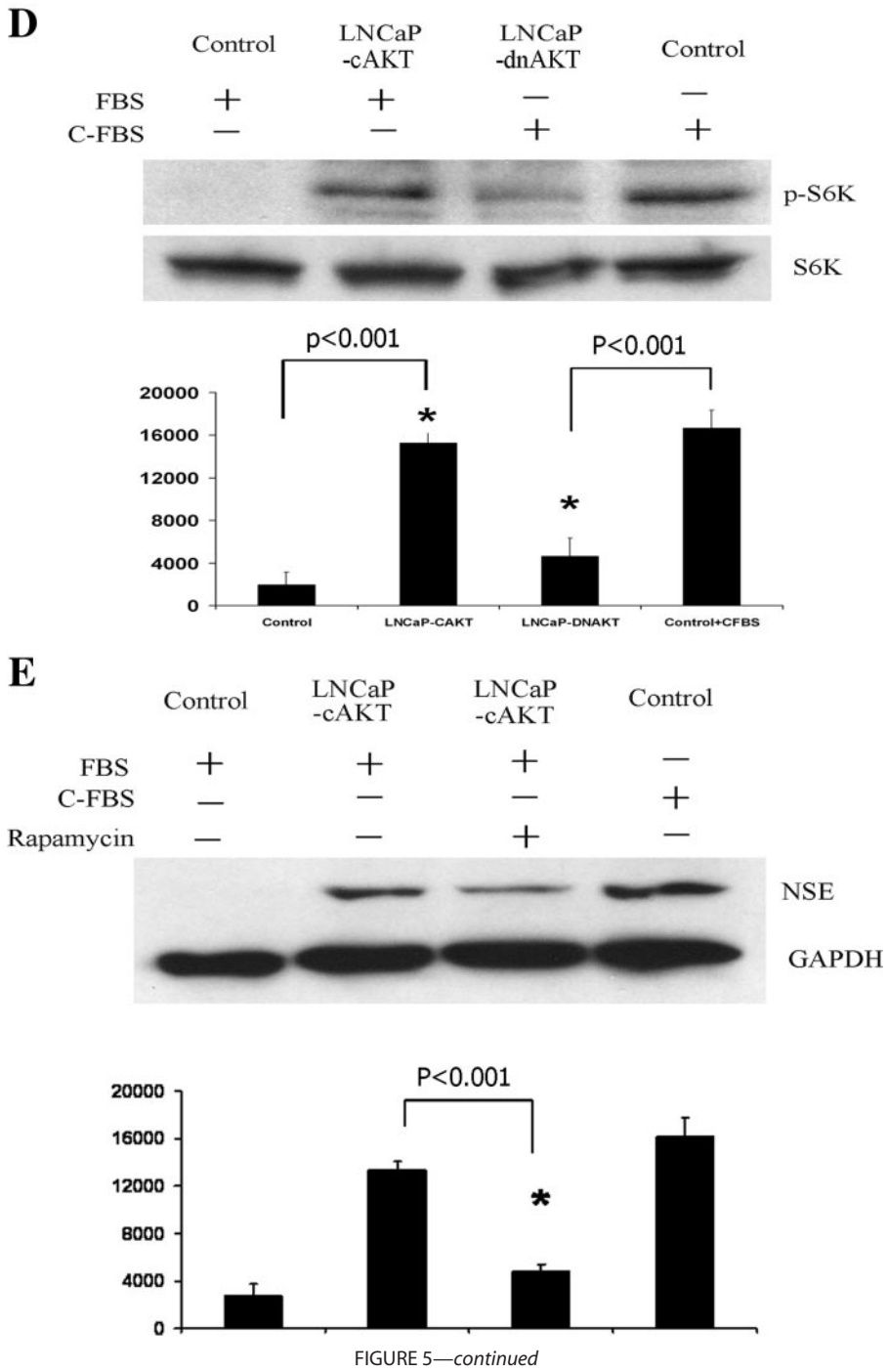


FIGURE 5—*continued*

prived media. LY294002, a PI3K inhibitor, and rapamycin, an inhibitor of mTOR, significantly inhibited the expression of NSE (Fig. 3*A* and Fig. 4*A*). The specificity of LY294002 was demonstrated by its ability to inhibit the phosphorylation of AKT (Fig. 3*B*) but not ERK phosphorylation (Fig. 3*C*). Similarly, we demonstrated that rapamycin inhibited the phosphorylation of S6K, a downstream molecule of mTOR (Fig. 4*B*), but not ERK (Fig. 4*C*). Additionally, we studied whether inhibition of AKT by a chemical inhibitor (AKT inhibitor IV) may inhibit NED. Because treatment of LNCaP cells with this compound for longer than 12 h causes significant cell apoptosis, we performed our study for 12 h (complete NED occurs in 6 days). LNCaP cells cultured in charcoal-treated FBS for 12 h showed increased NSE mRNA but not its protein (data not shown). We showed that AKT inhibitor IV abolished the increased expression of NSE mRNA after androgen withdrawal (Fig. 4*D*). Taken together, these results suggest that the PI3K-AKT-mTOR pathway, but not the Ras-MEK-ERK pathway, is required for androgen withdrawal-induced NED of PC.

Activated AKT Leads to NED of LNCaP Cells—Results from the above experiments suggest that the PI3K-AKT-mTOR pathway is activated in LNCaP cells after androgen withdrawal and is required for NED. We next studied whether activation of AKT by other means also promoted NED. We transiently transfected LNCaP cells with pcDNA3-cAkt, which expresses a constitutively active AKT with a deletion of amino acids 4–129 replaced with a consensus myristoylation domain, or pcDNA3-dnAKT, which expresses a dominant negative (kinase-deficient mutant) AKT (K179A). A real time PCR assay 48 h after transfection showed that expression of c-AKT, not dnAKT, induced the expression of NSE mRNA in LNCaP cells (Fig. 5*A*), suggesting that activated AKT induces NED, and the kinase activity of AKT is required for this function.

To confirm the results obtained with transient transfection experiments, we established stable cell lines of LNCaP cells. The LNCaP cells were transfected with pcDNA3-cAKT and pcDNA3dnAKT, respectively and selected with G-418. Cells that survived the selection were pooled and designated as LNCaP-cAKT and LNCaP-dnAKT cells, respectively. Western blot showed significant overexpression of AKT in these cells in comparison to the parental LNCaP cells (Fig. 5*B*).

LNCaP-cAKT cells had a significantly increased level of NSE in comparison with that in the parental LNCaP cells when cultured in normal FBS (Fig. 5*C*), suggesting that activation of the AKT pathway promotes NED of LNCaP cells. mTOR mediates the function of AKT in inducing NED because a downstream effector of mTOR, S6K, was constitutively activated in the LNCaP-cAKT cells cultured in normal FBS (Fig. 5*D*). Addition-

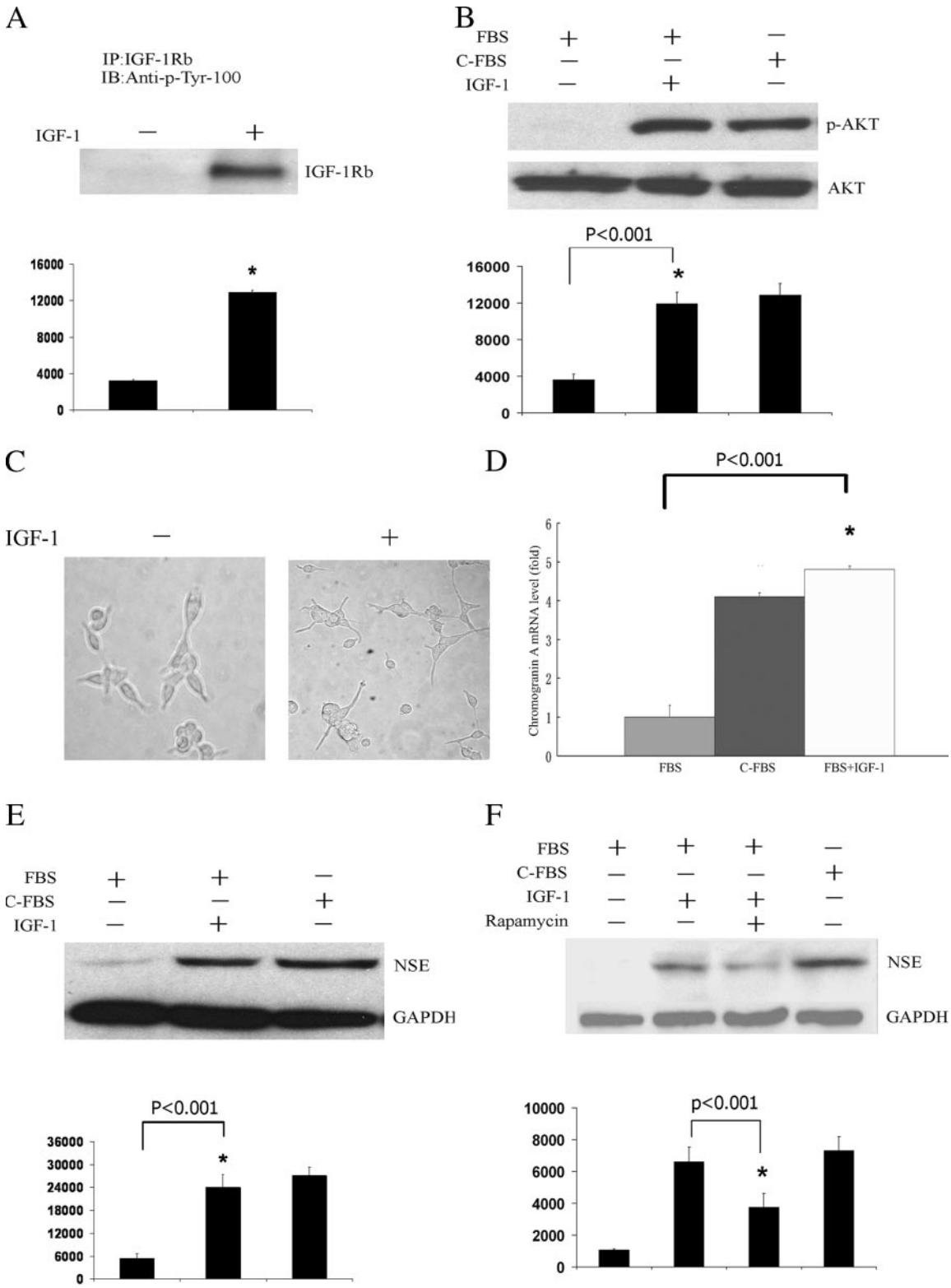


FIGURE 6. **IGF-1, an activator of AKT, induces NED of LNCaP cells.** *A,* LNCaP cells were cultured in FBS in the absence or presence of IGF-1 for 6 days. Equal amounts of proteins were immunoprecipitated (*IP*) with an anti-IGF-1-Rβ antibody and immunoblotted (*IB*) with an anti-phosphotyrosine antibody. *B,* LNCaP cells were cultured in FBS in the absence or presence of IGF-1 or in C-FBS (positive control of NED) for 6 days. Equal amounts of protein were immunoblotted with anti-AKT and anti-phospho-AKT antibodies. *C,* LNCaP cells were cultured in the absence (*left panel*) or presence (*right panel*) of IGF-1 for 6 days to show that IGF-1 induced NE-like morphology. *D,* LNCaP cells were cultured in FBS, C-FBS, or in FBS with IGF-1 for 6 days. A real time PCR assay was performed to study the expression of chromogranin A mRNA. *E,* equal amounts of proteins were immunoblotted with anti-NSE and anti-GAPDH antibodies to show that IGF-1 induces NED. *F,* LNCaP cells were cultured in FBS, C-FBS, or FBS plus IGF-1 in the absence or presence of rapamycin for 6 days. Equal amounts of proteins were immunoblotted with anti-NSE and anti-GAPDH antibodies to show that rapamycin inhibited IGF-1-induced NED. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.

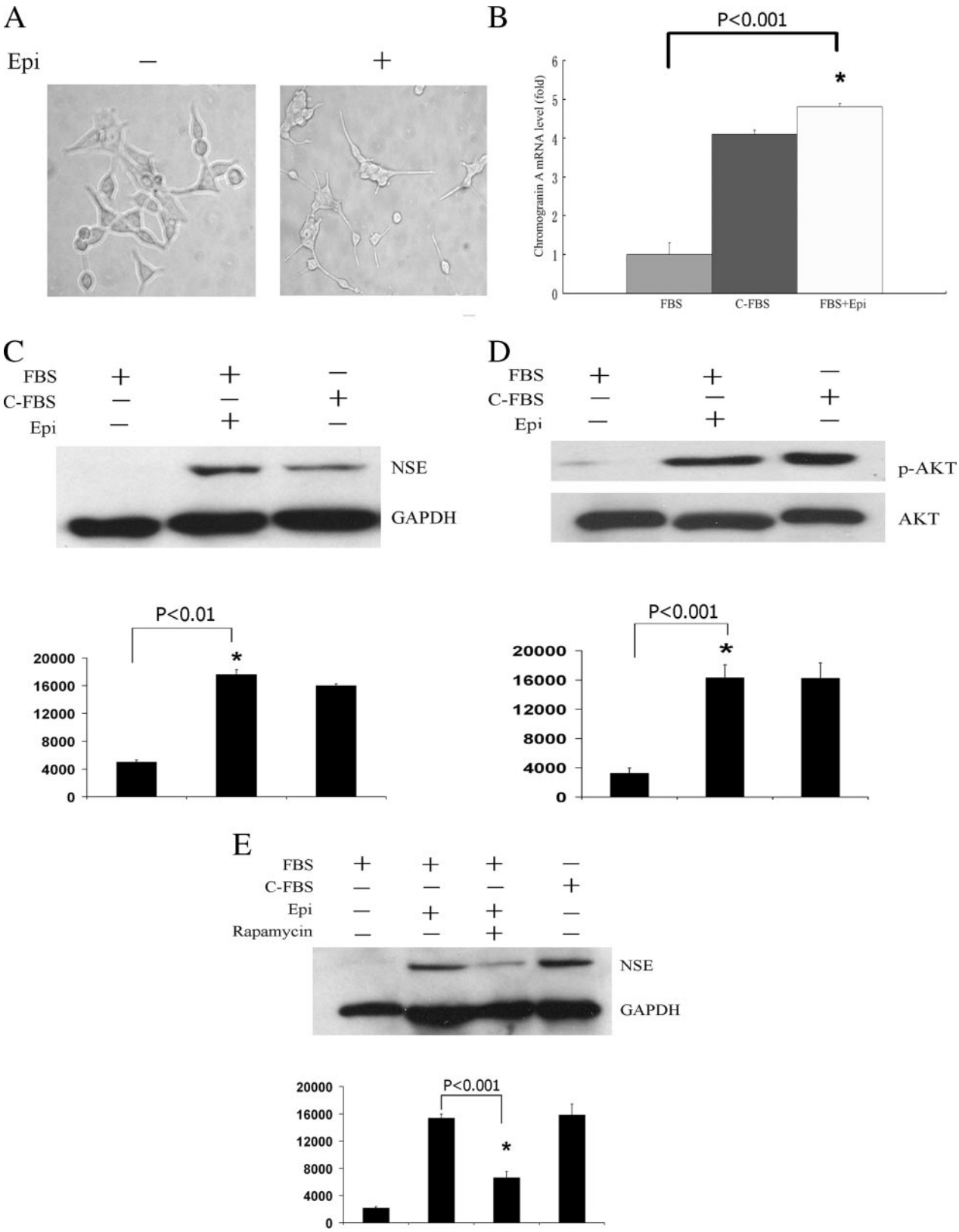
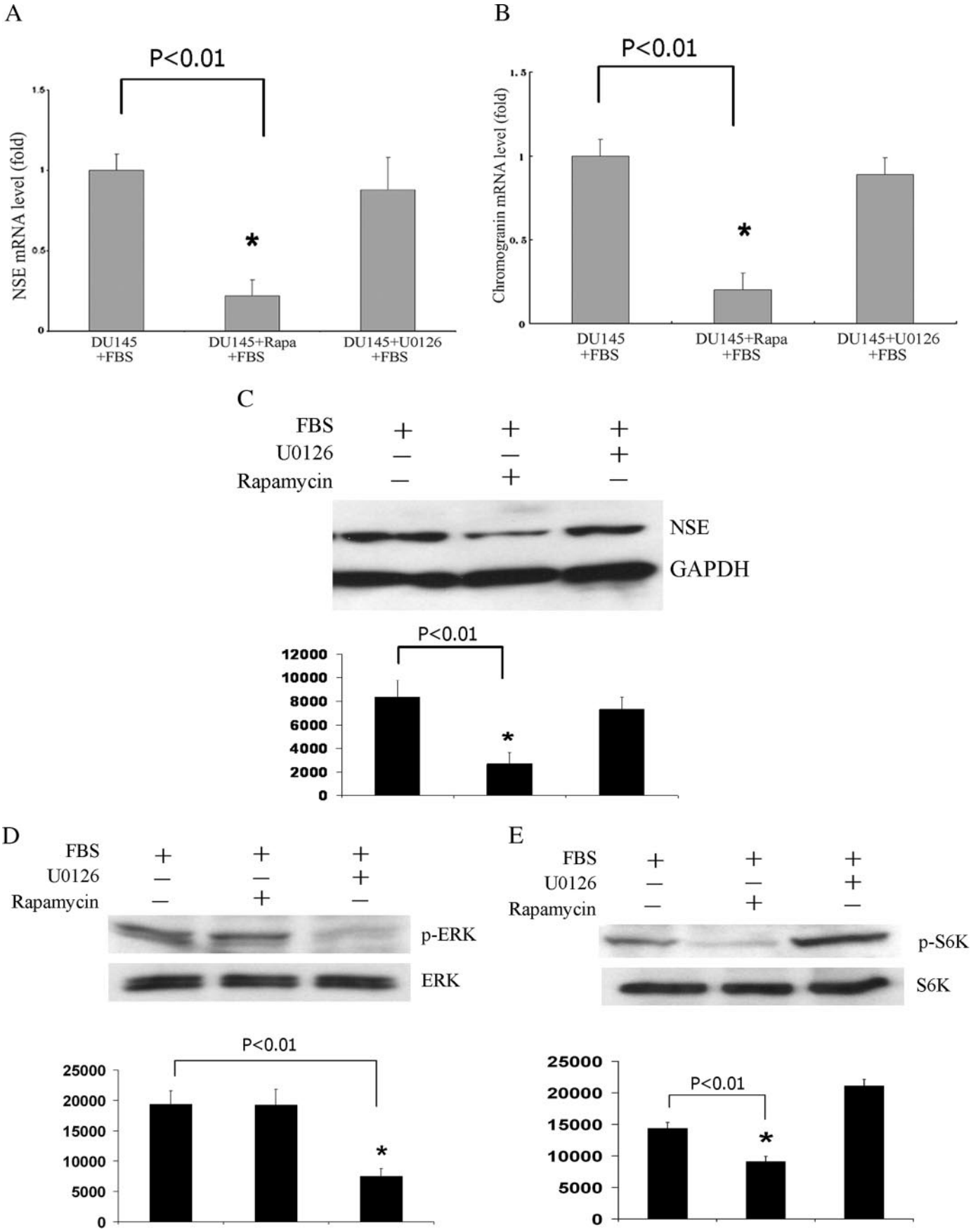


FIGURE 7. **NED induced by epinephrine (*Epi*) requires AKT signaling.** *A,* LNCaP cells were cultured in the absence (*left panel*) or presence (*right panel*) of epinephrine for 6 days to show that epinephrine induced NE-like morphology. *B,* LNCaP cells were cultured in FBS, C-FBS, or FBS with epinephrine for 6 days. A real time PCR assay was performed to study the expression of chromogranin A mRNA. *C* and *D,* Western blots show that epinephrine induced NED and also induced phosphorylation of AKT. *E,* LNCaP cells were treated with epinephrine for 6 days in the absence or presence of rapamycin. Untreated LNCaP cells cultured in FBS or C-FBS were used as negative and positive controls, respectively. Equal amounts of proteins were immunoblotted with an anti-NSE and an anti-GAPDH antibody to show that rapamycin inhibits NED induced by epinephrine. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.



ally, rapamycin, an mTOR inhibitor, inhibited NED of the LNCaP-cAKT cells (Fig. 5*E*). Expression of the dominant negative AKT significantly inhibited androgen withdrawal-induced NED (Fig. 5*C*) as well as phosphorylation of S6K (Fig. 5*D*), confirming that the PI3K-AKT-mTOR pathway is required for NED of PC.

IGF-1 Activates AKT and Induces NED of LNCaP Cells—We next studied whether activation of endogenous AKT by a stimulus other than androgen withdrawal may induce NED. We chose to study the effects of IGF-1, which activates AKT and has been implicated in the progression of PC in many studies (17). IGF-1 induced tyrosine phosphorylation of IGF-1 receptor β and the phosphorylation of AKT as expected (Fig. 6, *A* and *B*). Interestingly, addition of IGF-1 to LNCaP cells cultured in normal media induced NE morphologic changes similar to what is observed in cells cultured in androgen-deprived media (Fig. 6*C*) and also significantly increased the expression of chromogranin A and NSE (Fig. 6, *D* and *E*), suggesting that it induces NED. Importantly, NED induced by IGF-1 was inhibited by rapamycin (Fig. 6*F*), suggesting that IGF-1-induced NED is also mediated by the PI3K-AKT-mTOR pathway.

Epinephrine Activates AKT and Induces NED—Results from the previous experiments support the hypothesis that AKT is essential for the induction of NED of PC by androgen withdrawal. However, other agents, such as β-adrenergic receptor agonist epinephrine, can also stimulate NED of LNCaP cells (14, 18), but it was unclear whether AKT is involved under such conditions. Thus, we tested if NED of LNCaP cells induced by epinephrine also requires activation of AKT. Addition of epinephrine to LNCaP cells induced morphologic changes of NED and significant increases in the expression of chromogranin A and NSE as reported previously (Fig. 7, *A–C*) (14). Additionally, it also induced phosphorylation of AKT (Fig. 7*D*). Epinephrine-induced NED was significantly inhibited by the mTOR inhibitor rapamycin (Fig. 7*E*), suggesting that AKT also plays an essential role in this process.

NED in DU145 PC Cells Requires PI3K-AKT-mTOR Pathway—To confirm that the function of AKT signaling in NED is not limited to LNCaP cells only, we studied NED in DU145 PC cells. Unlike LNCaP cells that require androgen for proliferation, DU145 is an androgen-independent cell line that proliferates in the absence of androgen (19). Interestingly, unlike LNCaP cells, DU145 cells showed NED even when cultured in normal FBS as there were high levels of NSE and chromogranin A in such cells (Fig. 8, *A–C*). Similar to what was observed in LNCaP cells, rapamycin inhibited NED in DU145 cells whereas U0126 did not (Fig. 8*C*), confirming that PI3K-AKT-mTOR pathway, but not ERK, is required for NED. We confirmed the specificity of the inhibitors by showing that U0126 inhibited phosphorylation of ERK and rapamycin inhibited S6K phosphorylation (Fig. 8, *D* and *E*).

DISCUSSION

Progression of PC to the hormone refractory state is the major obstacle in PC therapy and has been an important focus of PC research. Many studies have shown that NED may play an important role in the progression of PC to AI state. In the transgenic adenocarcinoma of the mouse prostate model, NED is more extensive in poorly differentiated tumors and in recurrent AI tumors after castration (20). In the CWR22 human PC xenograft tumor model, castration induces an increase in tumor NE cells prior to tumor recurrence (21). LNCaP xenografts do not normally grow in castrated mice because they are androgen-dependent. However, they can grow in castrated hosts when NE cells from a mouse NE tumor (NE-10) are transplanted on the opposite flank, confirming that NE tumor cells can promote AI growth of PC (22). In the presence of androgen, the same NE cells can enhance migration and metastasis of PC cells (23).

We have shown that NE cells do not express AR (24). It is thus conceivable that hormonal therapy, which causes apoptosis of the AR-positive secretory cells, will not eliminate NE tumor cells. The NE tumors cells may then establish paracrine networks to induce AI proliferation of PC. Consistent with this hypothesis, we have shown that the NE cells in human PC secrete IL-8, a mitogenic and angiogenic factor for many tumors, including PC, and the surrounding non-NE tumor cells express increased levels of IL-8 receptor CXCR1 (25).

LNCaP is a very useful model because androgen withdrawal induces NED, mimicking *in vivo* situations in PC patients receiving hormonal therapy (13). With this model, we and others have shown that protein- tyrosine phosphatases may play a role in NED (26–28). Additionally, ERK (27, 29) has been reported to be important in NED. In this study, we were able to reproduce the previously published finding that androgen withdrawal in LNCaP cells induces the activation of ERK. However, in contrast to previous reports (27, 29), we found that inhibition of ERK activation did not suppress NED. One explanation is the possible difference in the chemical inhibitors used. The two previously reported studies used MEK inhibitor PD98059, while our study used the newer MEK inhibitor U0126, and there could be differences in the specificities of the two inhibitors. There may also be differences in other experimental conditions. For example, it has been reported that the LNCaP cells of different passage numbers show significantly different properties (30). For that reason, we performed all our experiments with LNCaP cells under passage 40.

AKT is an important signaling molecule in mammalian cells. It is activated by PI3K and inhibited by tumor suppressor gene PTEN (31, 32). Loss of function of *PTEN* is seen in some human PC cases (33). LNCaP harbors a point mutation in *PTEN* (34, 35), which may allow activation of AKT readily in such cells. We, as well as others (30, 36), have shown that the activity of

FIGURE 8. **Basal NED in DU145 cells requires PI3K-AKT-mTOR pathway.** *A–C,* DU145 cells were cultured in FBS, FBS + rapamycin, or FBS + U0126, respectively. Real time PCR assays and Western blot show that rapamycin, not U0126, inhibited basal NE differentiation. *D* and *E,* the relative specificity of the inhibitors is confirmed by showing inhibition of ERK phosphorylation by U0126 and inhibition of S6K phosphorylation by rapamycin. Results are plotted as mean ± S.D. from three replicates for each treatment group. Significant changes (compared with control) are indicated by an asterisk.

AKT in LNCaP cells is significantly increased after androgen withdrawal. Moreover, we have shown that activation of AKT after androgen withdrawal is inhibited by a PI3K inhibitor, suggesting that androgen withdrawal may activate a signaling molecule upstream of PI3K, such as a protein-tyrosine kinase or a G protein, leading to phosphorylation and activation of AKT. AKT participates in a variety of cellular processes, including proliferation, apoptosis, and survival, and is considered a key player in many tumors, including PC (37). Up-regulation/activation of AKT has been reported in a number of tumors, including PC (38–41). However, the mechanism of action of AKT in various cancers is not clear and is likely cell type- and organ-dependent. Transgenic mice expressing activated AKT in the prostate develop prostate intraepithelial neoplasia, a precursor lesion of PC, which is antagonized by the rapamycin analog RAD001, an inhibitor of mTOR (42). Similarly, in most other studies, AKT activation appears to be generally associated with malignant transformation and cell proliferation (33, 43, 44). Therefore, our observation that activation of AKT induces NED of LNCaP cells appears surprising at first because NE cells, having a differentiated phenotype, are generally nonproliferative (24). In support of our conclusion that AKT may have functions more diverse than originally anticipated, it has been shown recently that AKT actually blocks motility and invasion of breast cancer cell lines (45, 46).

It has been reported previously that AR may actively repress the NE phenotype of PC cells and inhibition of AR signaling leads to NED (47). It is therefore possible that activation of AKT may inhibit AR signaling, leading to NED. Consistent with this hypothesis, it has been shown that WNT signaling suppresses AR protein level (48) and induces NED in LNCaP cells (49). Similarly, HB-EGF, a ligand of the EGF receptor, can inhibit AR signaling (50) as well as inducing NED in LNCaP cells (29). It is possible that both wnt signaling and activation of EGF receptor by HB-EGF may stimulate NED of LNCaP cells through inhibition of AR signaling, similar to the effect of androgen deprivation of the culture media. Interestingly, the AR inhibitory function of HB-EGF also requires the PI3K-AKT-mTOR pathway but not the activation of ERK (51), supporting our conclusion that PI3K-AKT-mTOR pathway, not ERK, is critical in NED of PC.

Although our data indicate that the PI3-AKT-mTOR pathway, not ERK, is critical for NE differentiation, the true intracellular process may be more complex, and there may be cross-talk between the two pathways. For example, we have consistently observed increased phosphorylation of AKT after U0126 treatment in androgen-deprived LNCaP cells. Similarly, Zhuang *et al.* (52) reported that blockade of ERK1/2 by U0126 resulted in an increase in Akt phosphorylation in renal proximal tubular cells treated with H_2O_2. The potential cross-talk of the two pathways needs more investigation but that is beyond the scope of this study.

Our study shows that activation of the PI3K-AKT-mTOR pathway is necessary and sufficient for NED of PC. Activation of this pathway is required for NED of LNCaP cells induced by either androgen deprivation or epinephrine; and activation of the pathway by either overexpression of a constitutively active AKT or treatment with IGF-1 leads to NED. Inhibition of the pathway at the levels of PI3K, AKT, or mTOR all leads to inhibition of NED. In general, our data showed that inhibition of the pathway did not completely inhibit the increased level of NSE. This may be due to incomplete inhibition of the PI3-AKT-mTOR pathway as phosphorylation of the downstream molecule S6K was not completely inhibited in these experiments. Nonetheless, our study does not exclude the possibility that other pathways may also participate in the NED process.

Currently, multiple clinical trials are in progress targeting PI3K-AKT-mTOR signaling pathway in PC patients based on the observation that this pathway may be a key player in malignant transformation and cell proliferation (33). We show here for the first time that AKT is critically involved in NED of PC after androgen deprivation. This novel finding has important implications in interpreting the results of the clinical trials and should also help investigators in designing future treatment strategies for AI PC. It is reasonable to hypothesize that the combination of hormonal therapy, which induces NED through the PI3K-AKT-mTOR pathway, and an agent targeting the PI3K-AKT-mTOR pathway may suppress the proliferation of PC while inhibiting NED, thus possibly delaying/preventing the emergence of AI PC.

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Neuroendocrine-like cells in prostate cancer express CD44 and may represent the putative cancer stem/progenitor cells

Short Title:
Prostate cancer stem cells

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Abstract:
Androgen stimulates growth of prostate cancer (PC) via androgen receptor (AR). Androgen ablation therapy is therefore effective for advanced/metastatic PC. Unfortunately, the initial benefit is always followed by progression of the tumor to the hormone-refractory stage in which the tumor cells re-grow in the androgen-deprived environment.
It has been proposed that PC contains cancer stem cells (approximately 1-5% of the total tumor cell population) that renew themselves and give rise to differentiated cancer cells. The cancer stem cells do not express AR and are resistant to androgen ablation therapy and responsible for tumor recurrence. Therefore, identification and targeting of the cancer stem cells is the key to achieve the cure of PC. Patrawala et al (Oncogene, 2006; 25: 1696) reported convincing evidence that CD44+ cells in PC cell lines and xenograft tumors possess stem/progenitor properties. These cells are more proliferative, clonogenic, tumorigenic, and metastatic than the CD44- cells. They express 'stemness' genes, are AR- but can generate AR+ CD44- cells in-vitro and in-vivo. Some CD44+ PC cells can undergo asymmetric cell division.
Although there is strong evidence that CD44+ cells may represent PC stem cells, they have not been identified and chracterized in human PC tissue. We and others have shown that in PC tissue from patients, the tumor cells are heterogeneous. The majority of the tumor cells are the secretory type cells that express AR while 1-5% of the tumor cells are the neuroendocrine (NE)-like cells that do not express AR. The NE-like cells increase in high grade/stage PC, particularly after androgen ablation therapy and in hormone-refractory PC. In xenograft and genetic models of PC, castration increases the number of NE-like cells before tumor recurrence.
Since the NE-like PC cells fit the profile of the proposed cancer stem cells, we stained adjacent sections of human PC tissue with antibodies for CD44 and chromogranin A (CgA), a NE cell marker, and showed that in human PC tissue, CD44+ cells are the NE-like cells and vice versa. We performed double staining of human PC tissue microarray containing 200 cores of PC by immunofluorescence and showed that membrane CD44 staining and cytoplasmic CgA staining were always in the same tumor cells (1-5% of total tumor cell population). When PC cell lines LNCaP, DU145 and PC3 were sorted as CD44+ and CD44- cells by flow cytometry, expression of NE marker NSE was exclusively seen in CD44+ cells by western and/or real-time PCR assay.
Thus, our study has firmly established that in PC tissue from patients, the long-recognized NE-like cells

are the CD44+ cells which may represent the cancer stem/progenitor cells. We can now explain why every case of human PC contains a small population of NE-like cells that do not express AR and are resistant to hormonal therapy. Targeting NE-like PC cells should be a priority in designing future treatment strategies.
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Category and Subclass (Complete): TB02-05 Cancer stem cells
Primary Organ Site (Complete):
Organ Site Classification: Applicable. My abstract does refer to a specific primary organ site/tumor type as indicated below.
Primary Organ Site: Genitourinary cancers: prostate

Chemical Structure Disclosure (Complete):
Choose Chemical Structure Disclosure: NO.
Please explain (maximum 250 characters with spaces): : NA

Research Type (Complete): Translational research
Keywords/ Indexing (Complete): Prostate tumor ; Stem cells ; Neuroendocrine differentiation ; CD44
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Title: CD44 is a Useful Marker for Prostatic Small Cell Carcinoma

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Background: CD44 and its isoforms mediate epithelial cell adhesion and are implicated in cell proliferation and migration. Their levels of expression have prognostic value in certain malignancies. CD44 has attracted significant attention because of its potential as a stem cell marker in many tumors including prostate cancer. It has been reported that CD44 is expressed in basal cells of benign prostate but not in the majority of the prostate adenocarcinoma cells. Here we report the value of studying CD44 expression in the diagnosis of prostatic small cell carcinoma (SCC).

Design: Immunohistochemical staining was performed on 59 cases of SCCs using a monoclonocal anti-CD44 antibody (Santa Cruz, sc-7297, 1:1000) which recognizes CD44s and all isoforms. Origins of SCCs included prostate (12), lung (11), female genital organs (cervix/lower uterine segment/vagina/ovary, 14), bladder (10), head and neck (6), stomach (3), and pancreas (3). Staining was evaluated for the percentage of positive tumor cells and intensity. A case was considered positive only when there was strong membrane staining in greater than 50% of tumor cells. Statistical analysis was performed using Fisher Exact Test.

Result: As reported in the literature, CD44 staining was strong and diffuse in basal cells of the benign prostate but focal (<5%) in prostatic adenocarcinoma. SCCs of prostate were positive for CD44 in 92% (11/12) of the cases with strong and diffuse membrane staining. In contrast, only occasional cases were positive in SCCs of the lung (3/11, 27%), head and neck (1/6, 17%), and female genital organs (2/14, 14%). CD44 staining was negative in all other SCCs (bladder, 0/10; stomach, 0/3; pancreas, 0/3). The difference between prostatic SCC and bladder SCC was statistically significant ($p < 0.001$).

Conclusion: 1. Strong and diffuse membrane staining for CD44 is a feature characteristic of prostatic SCCs which distinguishes them from prostatic adenocarcinoma with a solid growth pattern.
2. CD44 expression is rare in SCCs of non-prostatic origin.
3. CD44 expression reliably distinguishes prostatic SCC from bladder SCC, which has important diagnostic value.